THE UNIVERSITY OF ALBERTA

STUDIES ON THE GROWTH OF AN ENTEROTOXIGENIC STRAIN OF STAPHYLOCOCCUS AUREUS DURING THE MANUFACTURE AND STORAGE OF CHEDDAR CHEESE

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled

STUDIES ON THE GROWTH OF AN ENTEROTOXIGENIC STRAIN OF *STAPHYLOCOCCUS AUREUS* DURING THE MANUFACTURE AND STORAGE OF CHEDDAR CHEESE

submitted by James W. Ramsahoye in partial fulfilment of the requirements for the degree of Master of Science.

To my wife Janet, and my son Patrick

In appreciation of their patience, understanding and encouragement.

ABSTRACT

Several cheese were made following standard procedure employed in the making of Cheddar cheese. Enterotoxigenic staphylococci were added at different stages in the manufacturing process.

The results showed that there was no production of enterotoxin in the various cheeses. During the ripening of the cheese over a period of 35 weeks the staphylococci decreased in numbers but at no time were they entirely eliminated.

The growth and survival of the organisms were studied in Trypticase Soy Broth at varying temperatures and pH. The results showed that the staphylococci were restricted in both growth and toxin production below pH 5. At higher temperatures (25 and 37C) and pH 5.0 and 6.0 the organisms grew well until the pH of the broth dropped, after which they began to die off. Under conditions of constant pH (5.0 and 6.0) growth was profuse.

The significance of these findings in relation to the production of Cheddar cheese is discussed.

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INTRODUCTION

Cheese has been implicated in food poisoning outbreaks as early as 1884. Vaughn (1884) in that year stated "It is well known that cases of severe illness follow eating of some cheese". He studied "poisonous cheese" or "sick cheese" following a report of three hundred cases of food poisoning due to cheese in Michigan, and although not familiar with staphylococcal food poisoning at the time, concluded that the causative agent was a chemical poison, tyrotoxicon, and not of bacterial origin. He further concluded that "this chemical poison might be generated by the agency of bacteria". Baker (1884) in the same year reported eight outbreaks of gastroenteritis due to cheese and stated that cheese from one factory over a thirteen month period had caused a great many people to become ill. In the following year Sternberg (1885) reported that he found "micrococci in the fluid cavities in the cheese" and concluded "It seems not impossible that the poisonous principle is a ptomaine developed in the cheese as a result of vital activity of the above mentioned micrococcus or some other micro-organisms which had preceded it and had perhaps been killed by its own products". Reed (1893) reported numerous cases of food poisoning in Ohio due to the consumption of cheese containing tyrotoxicon. At that time it was considered that spoiled milk used in the cheese making operation was the cause and that two cows

supplying the milk were sick. Barber (1914) demonstrated that attacks of gastroenteritis were caused by a toxin produced by staphylococci. Jordan (1917) mentioned that cases of cheese poisoning were relatively numerous but did not give any details and thus such cases cannot be looked upon as being caused by staphylococci or any other microorganisms. Stone (1943) in a review article mentions "Jack cheese" as being involved in a staphylococcal food poisoning outbreak. MacDonald (1944) described four cases of staphylococcal food poisoning in Great Britain from homemade goat's milk cheese. Tanner (1953) lists 21 cases in eight families in Germany as being due to staphylococcal enterotoxin in cheese, based on symptoms and epidemiological evidence. Mandry (1933) reported three outbreaks involving 18 persons in Puerto Rico in 1930 in which cheese was involved. Staphylococci were recovered from the cheese in each outbreak. Filtrates of organisms isolated from two of the outbreaks were given to human volunteers and produced symptoms similar to those of the original cases. Jordan (1930) in a report included results of studies on the organism isolated from one of those outbreaks. In reporting 183 food poisoning outbreaks of the "toxin" type that occurred in Great Britain during a ten year period Scott (1939) stated that three were due to cheese.

In the United States several outbreaks have been recorded during the years 1944 to 1952. _Staphylococcus_ _aureus_ was incriminated in 71 cases in Virginia in 1944. Examination

of cheese from an outbreak in Kentucky in 1945 showed hemolytic coagulase-negative staphylococci. A specimen of cheese from the same factory and the same lot revealed beta hemolytic coagulase-positive staphylococci. In the same year in Puerto Rico 17 cases were reported in which "native type" cheese made by a farmer in his home from milk from one of his cows was consumed. All persons who ate the cheese became ill including a family who purchased three ounces and distributed this among seven children, each child eating ten to twelve grams. One child aged four died. Laboratory examination of the cheese showed contamination with hemolytic *Staphylococcus aureus*. Again in Puero Rico in 1946 *S. aureus* was incriminated in three cases in which "native type" cheese was eaten. In 1947 cheese made at a monastery was responsible for 16 cases in four outbreaks in Kentucky. On examination of the cheese *S. aureus* was isolated. In 1948 three cases in Oregon were attributed to "cheddar type cream cheese" that had been held in an unrefrigerated show case for three months and from which hemolytic staphylococci were recovered. Dauer and Sylvester (1955) reported numerous cases of food poisoning due to homemade cheese,Cheddar cheese,cream cheese and cheese sauce all of which contained staphylococci. In 1958,sixty cases were reported from Indiana and Michigan due to Cheddar cheese produced in Wisconsin (1958). In that same year there was an outbreak of food poisoning at a State Institution in Iowa. Two hundred persons became ill and subsequent investigation incriminated beta hemolytic *Staphylococcus aureus*.

Sanitary inspection of the factory which made the cheese, equipment and staff showed negative tests for enterotoxigenic staphylococci. However, samples of milk from herds supplying the factory were obtained and coagulase-positive hemolytic _S. aureus_ were isolated from two of eight herds(1959).

There is thus abundant evidence mentioned above and elsewhere (Munch-Petersen (1960) and Report (1959)) in which cheese has been incriminated in staphylococcal food poisoning outbreaks. With such revelations coming forth an increasing awareness of the danger of cheese containing pathogenic staphylococci was recognised as was the significance of enterotoxin.

Nature of staphylococcal enterotoxin

Reports in the literature indicate that there are four serologically distinct types of enterotoxin (Casman 1958, Bergdoll _et al_.1959,Bergdoll _et al_.1965 and Casman _et al_.1966). They have been designated enterotoxin A, B, C, and D respectively and have been found to be closely related (Casman _et al_.1963). Purification procedures have been inadequate in determining the nature of these enterotoxins and it is only within recent times that some information with regard to this has become available. Enterotoxin A has been purified to 70% and enterotoxin B to 90% (Hopper 1963).

Enterotoxin B in the pure state when freeze dried appears as a fluffy white powder which is very soluble in

water. Hammon (1941) claimed the enterotoxin to be a complex carbohydrate but recent studies (Spero et al.1965) have shown it to be a simple protein containing 19 amino acids. Of the amino acids isolated aspartic acid,lysine,glutamic acid and tyrosine have been found to be present in large quantities (Bergdoll 1956). There appears to be some disagreement as to the correct molecular weight of the protein and two authors have estimated it at 24,000 (Hibnick and Bergdoll 1959) and 35,000 (Wagman et al.1965) respectively. The enterotoxin is heat stable and although some of the activity may be lost after boiling for 30 minutes it is still potent (Bergdoll et al.1951). The work of Read and Bradshaw (1965) has shown that the time-temperature combinations used in normal pasteurisation,sterilization and spray drying of raw milk are insufficient to reduce the level of the toxin. The enterotoxin can tolerate a wide range of pH values. It will withstand pH 3 - 12 for four hours at room temperature (Hunter and Dack 1938). These characteristics enhance the potency of the enterotoxin.

Detection of staphylococcal enterotoxin

It is generally agreed that the coagulase test is the best means of detecting potentially enterotoxic staphylococci(Evans and Niven 1950). The isolation of coagulase-positive staphylococci from a food product is taken as an indication of a potential source of enterotoxin (Allison 1949, American Public Health Association 1966 and Wilson

et al.1959)although all coagulase-positive staphylococci do not produce toxin. There is evidence to support the view that some coagulase-negative staphylococci can produce enterotoxin (Dack 1937). Thus, it is essential to detect enterotoxin in a suspected food product. Many selective media have been developed for the isolation of coagulase-positive staphylococci (Baird-Parker 1962, Hopton 1963, Crisley et al.1965, Jay 1963 and Raj 1966). As the effective agent in staphylococcal food poisoning outbreaks is the enterotoxin efforts have been made to discover rapid methods of detection of the enterotoxin and for testing its potency. Until recently detection of enterotoxin was quite a lengthy process but Weirether et al.(1966) have developed a rapid assay method which ought to give satisfactory results in one day. Animal and serological tests have been commonly used to detect enterotoxin. Other methods of detection such as infra-red spectrophotometry (Levi et al. 1956) and indirect haemagglutination (Robinson and Thatcher 1965) have been used.

(i) Animal tests

Man, rhesus monkeys, kittens and other animals have been used as biological agents for detection of enterotoxin. However, the reliability of these agents has not been uniform as there are variations amongst individual animals and also numerous inconsistencies. Kitten tests and feeding to monkeys have been criticized by Fulton (1943) and Elek (1959). There is also evidence that non-enterotoxigenic staphylococci

will cause food poisoning symptoms in cats and monkeys (Dack 1937).

(ii) Serological tests

With the purification of enterotoxin B and the determination that it was an antigenically active protein serological methods were being given more attention as a direct method to detect enterotoxin. Detection of enterotoxin is based on the principle that when mixtures of antigens diffuse into a gel containing mixtures of antibodies specific against the antigens there will be zones of precipitation. These zones of precipitation can be measured qualitatively and quantitatively. On this principle methods have been developed and become known as the Oudin gel diffusion test (Oudin 1948), Ouchterlony test (1949), Oakley test (Oakley and Fulthorpe 1953) and Crowle's modified micro-slide test (Crowle 1958). There have been modifications to these tests by various workers. Hopper (1963) has described a flotation system for the detection of staphylococcal enterotoxin in food, and the use of immunofluorescent antibodies to detect staphylococcal enterotoxin was studied by Genigeoris and Sadler (1966).

In comparison to animal tests the serological methods have been found to be reliable and sensitive with the only complication arising from the need for specific antiserums which require the use of pure toxins. The extraction of pure toxin is quite a laborious task and is also of a complex nature for routine work. Unless there is

readily available antiserum commercially, quick determination of enterotoxin serologically is almost impossible.

(iii) Effect of cheese infected with *S. aureus* on volunteers.

Roughley and McLeod (1961) reported that Cheddar cheese which was made with milk infected with coagulase-positive *S. aureus* was given to several people after ten weeks of storage. No ill effects were observed. Mattick *et al.* (1959) gave a quarter to a half of a pound of infected cheese to each of twelve adults, two of whom ate a quarter of a pound of cheese at one meal. None of the twelve persons suffered ill effects following consumption of the cheese. Although this evidence is not conclusive it does indicate that there was little or no enterotoxin produced in the cheese made or perhaps there was an alteration of the toxin, if any, causing a lack of activity.

Symptoms of staphylococcal food poisoning

Symptoms of persons who have ingested staphylococcal enterotoxin vary depending upon the amount of toxin ingested and also upon the individual. However, there is agreement in that some of the most common symptoms are nausea, vomiting, abdominal cramps, retching and diarrhoea. Any one or more of the symptoms mentioned may appear within 30 minutes to three hours after ingestion of the toxin (Thatcher 1966). Full understanding of the mode of action of the toxin is not quite clear but there is evidence to support the view that the toxin acts on the abdominal

viscera (Palmer 1951 and Sugiyama 1965).

Source of pathogenic staphylococci in Cheddar cheese

In reviewing the literature the main source of pathogenic staphylococci entering Cheddar cheese appears to be through the milk used for making the cheese (Reed 1893, Hendricks *et al*.1959, Thatcher and Ross 1960 and Sharpe *et al*.1962), such milk being the product of a herd having clinical or sub-clinical mastitis and carrying a strain of *Staphylococcus aureus* which is enterotoxigenic. It is possible, however, that contamination can be caused by handling of the product during the cheese making operation by a person or persons carrying enterotoxigenic strains in the nasopharyngeal region and on the hands. Adulteration,unclean equipment and utensils can also be a source. However, there has been no reported incidence in which sources other than milk have been held responsible.

Multiplication of staphylococci during cheese making

Using enterotoxigenic strains of *S. aureus* Takahashi and Johns (1959) infected raw milk before making Cheddar cheese. They found that the numbers increased during the cheese making operation. Furthermore, they established that most of the organisms were trapped in the curd. Mattick *et al*.(1959) inoculated pasteurized milk with two strains from mastitic herds. These strains produced dark zones on sheep-blood agar characteristic of β toxin. The amount inoculated gave

a bacterial content slightly higher than in practice. They got an increase of eight to 20 fold at the time of pitching the curd with little change at milling. Thatcher and Ross (1960) found that 0.03 I.U. of penicillin per ml in the milk restricted the multiplication of staphylococci in contrast to milk that contained no penicillin. The strain used was sensitive to penicillin and chlortetracycline. Roughley and McLeod (1961) in experiments with Australian Cheddar cheese got a 140 fold increase in numbers using five strains of coagulase-positive S. aureus inoculated separately into pasteurized milk used for making the cheese. This increase was recorded up to the time of hooping. Walker et al.(1961) in making Colby cheese inoculated raw milk with coagulase-positive S. aureus and got an increase of these organisms during the process. McLeod et al.(1962) varying the inoculum of a coagulase-positive S. aureus in pasteurized milk used to make Cheddar cheese found that the extent of multiplication varied from one to five fold during the manufacture of the cheese. Reiter et al.(1964) made Cheddar cheese in which two strains of S. aureus were used. One strain was isolated from a cow having mastitis and the other from Cheddar cheese responsible for food poisoning. To some of the cheese milk being used they added specific phages for the starters Streptococcus lactis and Streptococcus cremoris so as to produce a cheese which would have a lower acidity than normal Cheddar cheese. It was found that the staphylococcal count in the phage treated cheese increased rapidly during the

cheese making process, but in the untreated cheese milk the rate was about one-fifth that of the phage treated milk.

Conventional methods were used to make all of the cheeses in the above experiments. There is thus abundant evidence which shows that staphylococci compete with starter organisms and natural inhibitory products in the milk. They also multiply during the manufacture of the cheese but at varying rates.

Survival of staphylococci during ripening of Cheddar cheese.

Cheese milk that has been inoculated with staphylococci and used for making Cheddar cheese shows that at the time of hooping there is a higher count of these organisms than the original inoculum. This increase may continue for a short period of time (Reiter *et al*.1964), but thereafter there is a continual decrease during the ripening period. The rate of decrease is quite variable and dependent upon factors such as temperature of storage,pH,presence of other microorganisms,extracellular products,moisture content and salinity. Roughley and McLeod (1961) did not recover any coagulase-positive *S. aureus* from 30 platings each of 0.01 gram of cheese after ten or more curing weeks. At hooping there was 1.4×10^7 per gram of *S. aureus* present in the cheese. Mattick *et al*.(1959) got similar results after 14—22 weeks of storage at 59F. However, McLeod *et al*.(1962) report having a count as high as 4.6×10^7 per gram of *S. aureus* in one cheese after 179 days of storage. The count at the beginning of

ripening was 1.1×10^{8} per gram. Tuckey _et al._(1964) have found that the longer the cheese was aged the lower the numbers of _S. aureus_ became but at no time did the organisms disappear as a result of the aging process. Reiter _et al._(1964) have reported counts as high as 10^{7} per gram viable organisms (_S. aureus_) being present in Cheddar cheese after 72 weeks of storage. Takahashi and Johns (1959) found that there was a 90% decrease of coagulase-positive _S. aureus_ in cheese made from milk with a high initial Standard Plate Count whereas cheese made from milk with a very low SPC contained large numbers of staphylococci.

Effect of temperature on staphylococci during storage of cheese.

The effect of temperature on _S. aureus_ in infected Cheddar cheese has been reported by Tuckey _et al._(1964). They found that Cheddar cheese stored at 45F for 21 days had a _S.aureus_ count of 72 times the initial milk inoculum. On the other hand cheese stored at 50 and 55F for the same length of time showed an increase of only 16 and 11 times respectively of the initial inoculum. After storage for 26 weeks the _S. aureus_ count at 45,50 and 55F was eleven,three and two decimal five times respectively of the inoculated milk at zero time.

Effect of pH on staphylococci during the aging of Cheddar cheese.

Mattick _et al._(1959) reported that staphylococci die out rapidly at about pH 5.0 in cheese and that a

difference of 0.2 pH unit in the range pH 5.1-5.3 significantly affected the rate of reduction in numbers the least rapid decrease being in the cheese of higher pH. Sharpe _et al_.(1962) found that at pH 6.6 staphylococci multiplied and survived in much larger numbers and for longer periods, little decrease in numbers of viable organisms being found after six months. The failure of staphylococci to die out in cheese of an unusually high pH (5.5-5.8) has also been reported (McLeod _et al_.1962). Reiter _et al_.(1964) have shown that _S. aureus_ proliferate in Cheddar cheese in which acid development was reduced by the use of bacteriophage to restrict the lactic cultures.

Staphylococci in competition

Much work has been done recently on the growth of staphylococci in the presence of other bacteria. Troller and Frazier (1963) have shown that the growth of _S. aureus_ was suppressed by _Serratia marcescens_ and _Pseudomonas_ sp. and the main factor of inhibition was competition for nutrients. In that same work it was shown that _B. cereus_, _Proteus vulgaris_, _E. coli_ H52, _Aerobacter aerogenes_ and _Achromobacter_ sp. inhibited _S. aureus_ by means of antibiotic substances. Graves and Frazier (1963) have shown some _E. coli_ cultures to be stimulatory on the growth of _S. aureus_ and also that other _E. coli_ were inhibitory. In a study of staphylococcal growth and enterotoxin production in meat Casman _et al_. (1963) have shown that staphylococci were unable to compete with other organisms and that the anaerobic conditions were

more effective in this inhibition. They also found that when the surfaces of raw and cooked meats were inoculated, however, good growth was obtained with the production of enterotoxin. Iandole _et al_.(1965) in studies of suppression of _S. aureus_ in associative culture found that _Streptococcus diacetilactis_ inhibited the growth of _S. aureus_ due to nutrient depletion. It was also established that _S. diacetilactis_ metabolized nicotinamide much faster than _S. aureus_. Ingram (1960) in studies of bacterial multiplication in packed Wiltshire bacon suggests that salt or nitrite in the bacon can prevent toxin formation and that psychrophiles crowded out the staphylococci when the bacon was held at low temperatures. More recent studies by DiGiacinto and Frazier (1966) have shown that coliforms and _Proteus_ repressed the growth of staphylococci. The effect of lactic acid bacteria on the growth of _S. aureus_ studied by Kao and Frazier (1966) showed that lactic acid bacteria were stimulatory in some cases and some were stimulatory at higher temperatures. Some lactic acid bacteria were inhibitory at lower temperatures and some were inhibitory at all temperatures employed (10-37C). Some killed staphylococci by development of acid at a later stage. McCoy and Faber (1966) have found that inhibition of _S. aureus_ by food microorganisms was more common than stimulatory. There was inhibition of growth and inhibition of enterotoxin production with no apparent effect on growth. It was found that _B. cereus_ stimulated significantly staphylococci to grow and to produce enterotoxin. _B. megaterium_ and _Brevibacterium linens_

some attention to this inhibition. They also found that the inhibition of lactic acid bacteria was proportional, however, acid production was associated with the concentration of bacteria. Daniels et al.(1963) in studies of association of L. bulgaricus in associative cultures with Streptococcus thermophilus indicated the growth of L. bulgaricus was adversely inhibited. It was also established that S. diacetylactis stimulated significantly more faster than S. lactis. Reeves (1962) in studies of bacterial contamination in market milk(dairy, found probably that gram positive and gram negative bacteria were more psychrophilic than lactic organisms and the psychrophilic than the lactic acid bacteria at low temperatures. More recent studies by Babel and Pearson (1966) have shown that coliforms and Pseudomonas re-pressed the growth of streptococci. The effect of lactic acid bacteria on the growth of S. aureus studied by Lee and Frazier (1966) showed that lactic acid bacteria were stimulatory (increase rates) and were more stimulatory at higher temperatures. Some lactic acid bacteria were inhibitory at lower temperatures and more were inhibitory at all temperatures employed (10-37C). Some lactic streptococci by development of acid at a later stage. Gilliland and Speck (1969) have found that inhibition of S. aureus by lactic streptococci was more severe than stimulation. There was inhibition of growth and inhibition of enterotoxin production but with no apparent effect on growth. It was found that S. aureus stimulated significantly streptococci to grow and at

were inhibited by staphylococci.

It is evident from the literature reviewed that Cheddar cheese has been responsible for numerous outbreaks of staphylococcal food poisoning. Numerous experiments have been carried out with coagulase-positive and enterotoxigenic strains of Staphylococcus aureus being added to raw and pasteurized milk used for making cheese. Anyone familiar with the manufacture of Cheddar cheese knows that the process involves handling of the curd at different times throughout the operation. It was decided that experiments should be carried out in which a strain of S. aureus known to be enterotoxigenic would be added to the cheese milk at various stages in the manufacturing process. The approach was taken that there is always a chance of contamination from workers, equipment and from the milk, and should such contamination occur what would be the effect on the organism involved and on the cheese ? The environment of the test organism would be such that the organism was in competition with other bacteria and chemical changes which go on in the manufacture and ripening of Cheddar cheese. Determinations on the growth and survival of S. aureus in the cheese would be made over a period of time. Data relating to the cheese making process would be reported and also tests would be carried out to detect enterotoxin production in the cheese made. It is pertinent to mention here that other workers have carried out experiments with Cheddar cheese in which an enterotoxigenic

strain was used but no mention has been made of efforts to detect enterotoxin following the making of such cheese. Some information on this aspect was therefore considered essential.

MATERIALS AND METHODS

Test organism

Cultures of Staphylococcus aureus strain Cas 243, A.T.C.C.14458, freeze dried onto porcelain beads, were grown out in double strength brain heart infusion broth (Baltimore Biological Laboratories) at 37C for 24 hours. This strain is known to produce enterotoxin B (Belfield 1966). The stock cultures were transferred to meat infusion agar slants. After growth at 37C for 24 hours the slant tubes were sealed and kept refrigerated at 4C.

Preparation of cultures of S. aureus Cas 243

2000 ml of double strength brain heart infusion broth were prepared and 200 ml quantities dispensed into nine 500 ml flasks. The flasks were then stoppered and covered with aluminum foil and sterilized in an autoclave for fifteen minutes at 121C and 15 pounds pressure. From a meat infusion slant a loopful of culture was transferred to one of the flasks. This was then placed on a Gyrotory Shaker (New Brunswick Scientific Co.,New Brunswick,N.J.) and incubated at 37C for 24 hours. Following this, serial dilutions were made and bacterial counts determined using Mannitol Salt Agar (Bacto) with incubation at 37C for 24 hours. This experiment, from taking a loop from the agar slant to making serial dilutions and plating was repeated twice. An average of total counts made showed that there were $100\text{-}115\times10^6$

organisms per ml. From this information it was decided that 200 ml of a 24-hour culture of S. aureus Cas 243 would give the desired inoculum of 10^5-10^6 organisms per ml in the cheese milk.

Preparation of inoculum

200 ml of a 24-hour culture of S. aureus Cas 243 was centrifuged in a Sorval RC2-B automatic refrigerated centrifuge. The supernatant was discarded and the cells resuspended in 20 ml of 0.2M phosphate buffer. The suspension was recentrifuged and the process repeated two more times. The cells were finally suspended in 100 ml of sterile distilled water.

Starter used in the manufacture of Cheddar cheese

The starter culture used for making the experimental cheeses was obtained from Klenzade Products,Beloit,Wisconsin. The culture used contained three or more strains of Streptococcus lactis or Streptococcus cremoris together with compatible citric acid fermenters (flavor and aroma producing bacteria).

Preparation of starter - stock culture

Skim milk was prepared by mixing 100 g of skim milk powder into 1000 ml of distilled water. After dissolving the powder the milk was dispensed in 100 ml aliquots into 200 ml flasks. The flasks were then stoppered with cotton wool, covered with aluminum foil and autoclaved for fifteen minutes

at 10 pounds pressure and a temperature of 115C. Two flasks, after being allowed to cool, were each inoculated with 100 mg of Klenzade lactic culture #2. One flask was incubated at 22C and the other at 32C. After 18 hours of incubation the flasks were examined for coagulation. It was noticed that the flask incubated at 32C had a thicker and smoother curd than the flask which was incubated at 22C. The acidity developed in the flask incubated at 32C was much more than the flask incubated at 22C. This treatment of the skim milk with lactic culture and incubating at two different temperatures was repeated twice. The results obtained were the same as before. The incubation temperature of 32C was used for preparing the stock culture. This temperature of incubation is unusual for starter organisms but as this particular culture performed better at 32C it was decided to use this temperature. Each day one ml of lactic culture prepared the day before was transferred to 100 ml of sterile skim milk. The stock culture was then placed in a cooler at 4C.

Source of milk supply

Milk for the experimental cheeses was obtained from the University Livestock Farm, University of Alberta, Edmonton. The milk was collected in the morning of the day of the cheese making operation, except in one case when it was collected on the evening before. The milk obtained was of high bacteriological quality with total counts not exceeding 30,000 organisms per ml.

Preparation of cheese

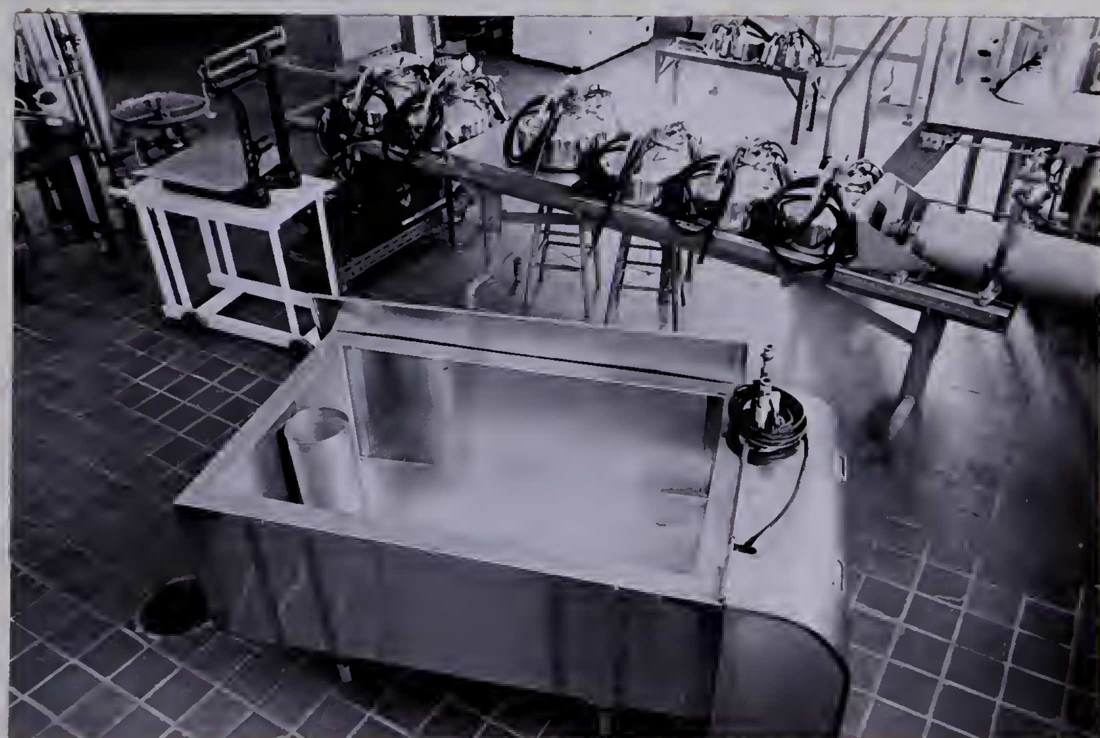
About 475-600 pounds of milk were used to prepare the cheese. Relevant data relating to titratable acidity,pH and total bacterial count were recorded before the operation. Using a selective medium, Mannitol Salt Agar (MSA), it was found that no pathogenic staphylococci were present in the cheese milk.

The milk was placed in a Cherry-Burrell Laboratory vat (Plate I) and the temperature raised to 86-88F (30-31.2C) after which the starter culture was added to give an inoculum of 1.5-2.0 per cent. The milk was then stirred with a rake (Plate II,E) for three minutes to incorporate and distribute the additives uniformly. The milk was held at this temperature for about one hour after which time rennet extract was added. The amount added was based on the addition of 85.2 ml per 1000 pounds of milk as carried out in commercial practice. After stirring, the vat was covered and the milk allowed to set. The curd was examined for firmness after setting for 30-40 minutes. This was done by inserting the right forefinger into the curd and slowly raising it when the curd was seen to break cleanly and the whey escaped freely from the broken surface. The curd was cut into uniform sized cubes using curd knives (Plate II,B and C). After cutting the curd the temperature of the vat was raised slowly at first and then rapidly to 102F (39C). This phase of the operation is known as the "cooking process" and was carried out for about 135-150 minutes. During this period samples were taken inter-

mittently to determine the change in titratable acidity.After cooking the curd became firm and when squeezed in the hand and then released it fell apart. Dipping of the whey followed cooking. The remaining curd was then levelled off to a fairly uniform height and a channel made in the centre for a more thorough leaching of the whey. The curd was left in this position for about 25-30 minutes and then cut sideways with a stainless steel knife (Plate II,A) into four slabs and piled into two layers. The curd was left in this position for about 20-25 minutes. The curd was then turned and allowed to remain in this position for about 30 minutes. The curd was then cut into strips about six inches wide and the strips turned over to allow a more complete release of whey. The strips were left in this position for about 20 minutes after which the curd was ready for milling.

The curd was milled in a hand operated cheese mill (Plate III). The milled curd was treated with about 1.5% salt and the whole stirred with a fork (Plate II,D). The salted curd was then placed into 20 pound hoops lined with disposable rayon press cloths which were previously soaked in a cold solution of sodium hypochlorite (200 ppm). Twenty-one pounds of curd were put into each hoop. The hoops were then placed in an hydraulic press overnight under a pressure of 40 pounds. The following morning the press cloths were removed, the cheese trimmed,weighed and samples cored out for testing. The cheese was then wrapped in Parakote film (Marathon Corp., Menasha,Wisconsin) and replaced into the hoops. The hoops were returned to the press and hot water poured over them to

(a)



(b)

Plate I. Cherry-Burrell Laboratory cheese vat

(a) With cover on

(b) With cover off showing circular strainer in whey exit

complete the sealing. The cheese was allowed to remain in the hoops for twelve hours. After this the cheese was packed in carton boxes and placed in a chamber at 50F (10C) for curing.

Any curd remaining in the vat after hooping was placed in a metal container and autoclaved to destroy all micro-organisms. After this treatment it was discarded. The vat was cleaned with steel wool and soap. Bits of curd adhering to the walls were removed thoroughly. The vat was then sprayed with very hot water and steamed. After this it was filled with warm water and sodium hypochlorite added to give a concentration of 300-400 ppm. Other equipment used was treated similarly and soaked in the hypochlorite solution in the vat for about 20 minutes. The solution in the vat itself was allowed to remain overnight after which the vat was drained and rinsed with hot water.

A total of six cheeses were made according to the above procedure with minor variations. Staphylococci were added before or during the manufacturing operation according to the scheme in Table 1.

Table 1. Scheme for preparing experimental cheese

Cheese	Time of addition of *S. aureus* Cas 243
A	Together with the starter
B	At salting
C	Control - no staphylococci
D	At rennetting
E	At start of cooking
F	12 hours before starter(milk held at 58F)

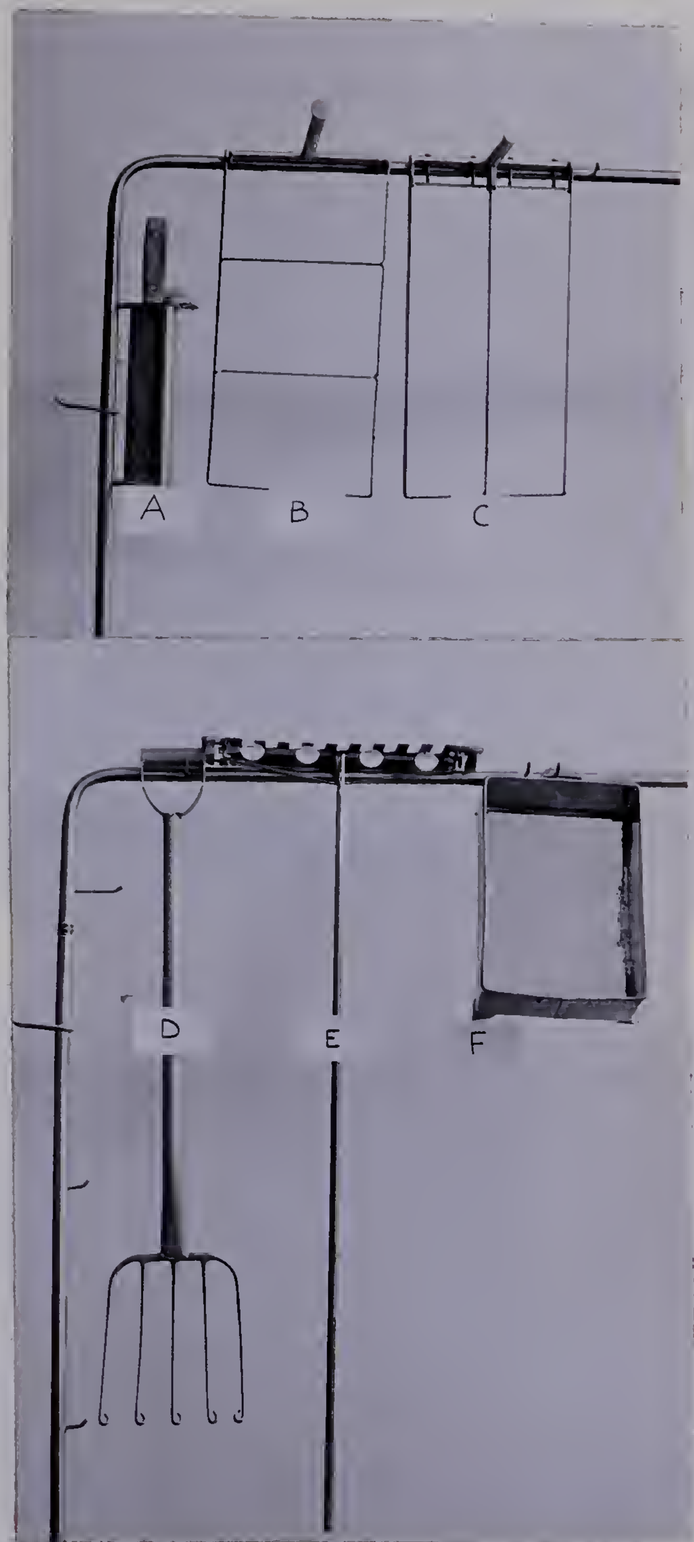


Plate II. Some equipment used in the manufacture of Cheddar cheese.

A - Stainless steel knife used to cut curd

B - Horizontally strung knife used to cut soft curd

C - Vertically strung knife used to cut soft curd

D - Fork with hooked in tips

E - Rake

F - Rectangular strainer

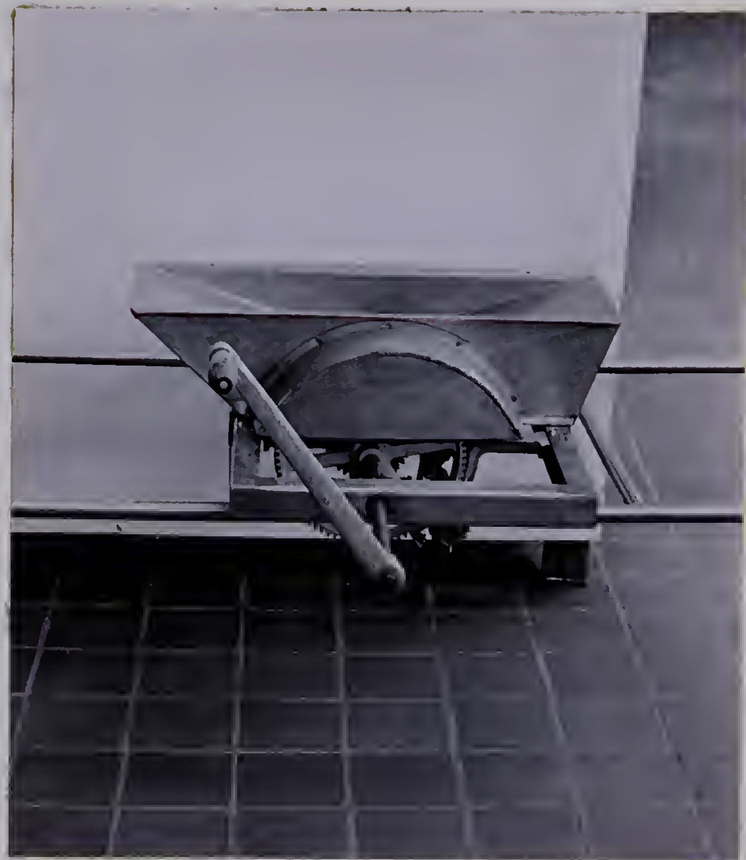


Plate III.(Above) Hand operated mill for milling cheese

(Below) Two of the experimental cheeses prepared

Enumeration of bacteria in cheese during storage

In carrying out the determinations samples were cored out of each cheese that was hooped. About 20 g of each cheese was also kept in storage at at 0C for reference. Twenty grams of cheese were weighed out and placed in a sterile Waring blendor. To this was added 180 ml of sterile distilled water. The blendor was covered and the mixture blended for about five minutes until homogenous. Serial dilutions were made of the suspension and plated in duplicate in both SPCA and MSA. Plates made with SPCA were incubated at 32C and those made with MSA at 37C, both for 48 hours.

Determination of pH of cheese during storage

Determinations of pH were made by mixing some of the cheese (about 5 g) with enough distilled water (pH 7.0) to form a paste. The instrument used to measure the pH was a Compensator E 322 (Metrohm Ltd., Herisau, Switzerland).

Physical examination of cheese

A physical examination of the cheese was carried out each day that bacterial enumerations were made. This examination involved observing the appearance, texture, surface contamination and smell of the cheese.

Serological methods for detection of enterotoxin.

The test used for detection of enterotoxin B in the cheese was a modification of the Oudin method of detecting

antigens as developed by Weirether *et al.*(1966). Detection of enterotoxin is based on the principle that when mixtures of antigens diffuse into a gel containing mixtures of antibodies specific against the antigens there will be many zones of precipitation. These zones can be measured qualitatively and quantitatively (Fig.1).

(i) Preparation of assay tubes

"Kimax" tubes 6 mm in diameter and 50 mm in length were internally coated with 0.5% Ionagar to prevent leakage at the glass - agar interface. This was achieved by using a "Plastipak" 2½ cc sterile disposable plastic syringe. The tubes were filled with agar and the agar slowly withdrawn thus coating the sides. Any excess agar was shaken out with a quick flick of the wrist. The tubes were then dried under vacuum (22" pressure) over a dessicant in a desiccator.

(ii) Preparation of antiserum agar

Antiserum agar was made from the following ingredients:-

0.02M phosphate-buffered saline

1% Ionagar

Specific antiserum for enterotoxin B

(a) Preparation of 0.02M phosphate-buffered saline

0.02M phosphate-buffered saline was made by mixing 85 parts of solution I and 15 parts of solution II.

Solution I was made up as follows:-

5.680 g of disodium phosphate were weighed out and dissolved in distilled water and made up to 600 ml.

17.00 g of sodium chloride were weighed out and dissolved in distilled water. To this was added 0.20 g of Merthiolate (Eli Lilly & Co.,Indianapolis,U.S.S.) and the mixture made up to 1400 ml. The disodium phosphate solution was then mixed with the sodium chloride solution,transferred to a storage bottle,shaken and stored at 4C until ready for use.

Solution II was made up as follows:-

2.70 g of potassium dihydrogen phosphate were weighed out, dissolved in distilled water and then made up to 300 ml. 8.50 g of sodium chloride were weighed out and dissolved in distilled water. To this was added 0.10 g of Merthiolate and the mixture made up to 700 ml. The two solutions were then mixed,transferred to a storage bottle,shaken and stored at 4C until ready for use.

When solutions I and II were mixed in the required proportions the pH of the buffer was 7.4

(b) Preparation of 1% Ionagar

5 g of Ionagar (Oxoid) were weighed out and added to495 ml of 0.02M phosphate buffer. The mixture was then heated for 10 minutes at 121C and after this filtered through a Whatman No.1 filter paper. Filtration to clearness was accomplished by holding at 55C during filtration.

(c) Preparation of 0.5% Ionagar

25 ml of 1% Ionagar was mixed with 25 ml of 0.02M phosphate buffer. The 1% Ionagar was in the fluid state before mixing. This 0.5% Ionagar was used to coat the "Kimax"tubes.

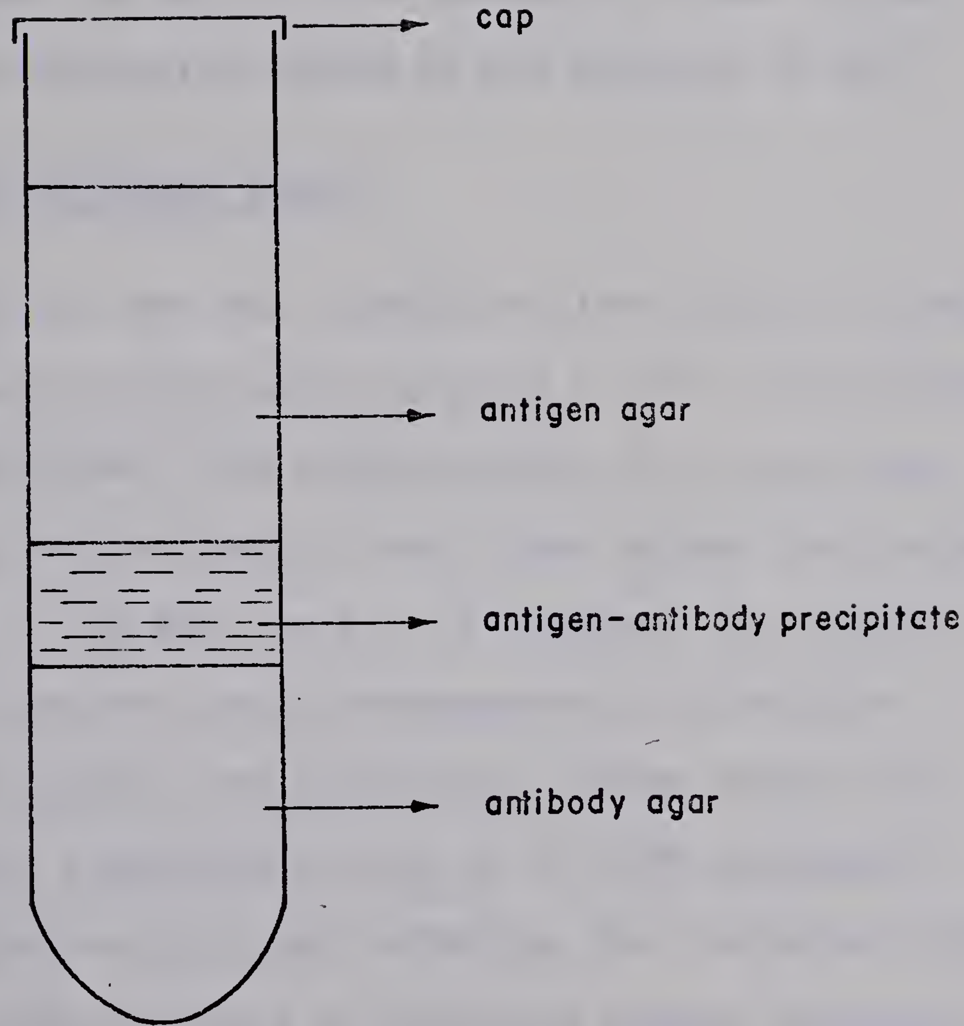


Fig.1 Single diffusion tube method for determination of enterotoxin

Preparation of antiserum agar (1:40 dilution) was made using the following procedure:-

9.5 ml of phosphate buffer was transferred to a sterile culture tube and placed in a water bath at 48C for fifteen minutes. To this was added 0.5 ml of specific antiserum. The tube was swirled to mix the ingredients thoroughly. Heated 1% Ionagar was allowed to cool to 48C. 10 ml of Ionagar was added to the culture tube and the whole thoroughly mixed. A sterile $2\frac{1}{2}$ cc disposable plastic syringe was used to add 0.35 ml of this antiserum agar to each of the prepared "Kimax" tubes. The tubes were covered with parafilm and allowed to set.

(iii) Preparation of crude toxin

S. aureus Cas 243 was inoculated into double strength brain heart infusion broth and incubated at 37C for 14 hours. The culture was divided into approximately 20 ml portions and placed into sterile plastic centrifuge tubes. The tubes were centrifuged at 10,000 rpm for 15 minutes, the supernatant discarded and the cells resuspended in distilled water. This was repeated two more times, after which the cells were finally suspended in 2.0 ml of 0.2M phosphate buffer for inoculation into sac cultures. Sac cultures were prepared from 7-inch sections of dialyzer tubing (porosity 48 Å, diameter 1.875 inches) (No.4465-A2 Arthur Thomas Co., Philadelphia,U.S.A.). The tubing was soaked in distilled water to facilitate handling, after which it was knotted at one end in two places. The tube was then filled with distilled water and tested for leakage. The sac was placed in a 300 ml

flask and 100 ml double strength brain heart infusion broth added to the sac. 18 ml of 0.2M phosphate buffer was then added to the flask outside of the sac. The sac was banded with rubber bands which assisted in fixing the sac to the top of the flask. The flask was plugged with cotton wool wrapped in aluminum foil, and sterilised. 2.0 ml of prepared inoculum was added to the buffer outside the sac and the flask placed in a Waterbath Shaker (New Brunswick Scientific Co.,New Brunswick,N.J.) at 37C and allowed to shake for 72 hours. The fluid outside of the sac was then collected in a sterile plastic centrifuge tube and centrifuged at 10,000 rpm for 15 minutes. The supernatant obtained was used as a source of crude enterotoxin.

(iv) Extraction of enterotoxin from cheese

The method used for the extraction of enterotoxin from the cheese was that developed by Read *et al*.(1965). The chemicals used for this extraction were 0.04M Veronal buffer, 3N sodium hydroxide and 6N hydrochloric acid. A 0.04M solution of Veronal buffer was made by dissolving 8.2472 g of Veronal buffer salt in sterile distilled water, adjusting the pH to 7.2 with 6N HCl, and making up to a litre.

40 g of each cheese were blended with 40 ml of 0.04M Veronal buffer in a sterile Waring blendor. The blended cheese was placed in a plastic centrifuge tube and centrifuged in a Sorval RC2-B refrigerated centrifuge at 15,000 rpm at 0C for 30 minutes. After centrifuging the supernatant was filtered through a Whatman no.12 filter paper and collected

in a 50 ml beaker. The fluid was acidified to a pH of 4.5 using 6N HCl, after which it was centrifuged at 10,000 rpm for 10 minutes. The supernatant was then collected and the pH adjusted to 7.2 with 3N NaOH. After this the solution was placed in a water bath at 50C for 10 minutes and then placed in a 13 ml Pyrex glass tube, centrifuged at 15,000 rpm for 30 minutes, and the supernatant placed in a sterile culture bottle. Two drops of Merthiolate (conc. 1:10,000) were added to act as a preservative. The tubes were then stoppered and stored at 4C until ready for assay.

(v) Application of test antigen

Appropriate holes were made in polyfoam boards to accomodate the assay tubes containing antiserum agar. The tubes were taken out of storage and allowed to sit at room temperature for abour two hours. Toxin extracts,crude toxin and control solutions were treated the same way. To the assay tubes was added 0.35 ml of toxin extract or crude toxin or control materials. The tubes were covered with parafilm, incubated at 30C and observed at 24, 48 and 72 hours. To eliminate any side reactions the following controls were prepared:-

No toxin	: no serum (0.70 ml 1% Ionagar)
Crude toxin	: no serum
No toxin	: serum
0.2M phosphate buffer	: no serum
0.2M phosphate buffer	: serum
0.02M buffer	: no serum
0.02M buffer	: serum

Merthiolate	: no serum
Merthiolate	: serum
Sterile broth	: no serum
Sterile broth	serum

Coagulase activity

Plugs of cheese were extracted and blended with sterile distilled water. Appropriate dilutions were made and plated on MSA. Two colonies from each plate were picked off,inoculated into sterile brain heart infusion broth, and incubated at 37C in a Gyrotory shaker for 24 hours.

To 1 ampule of coagulase plasma (Difco) was added 3 ml of sterile distilled water and the mixture swirled to mix thoroughly. 0.5 ml of this mixture was dispensed into each of five sterile ampule tubes with a sterile disposable syringe. Two drops of the 24-hour culture were added to each ampule. A control ampule was treated with two drops of sterile brain heart infusion broth. The ampules were then incubated at 35C and checked for coagulation after one hour.

Moisture content of cheese

The method as outlined in the Laboratory Manual,Milk Industry Foundation (1959) was used to determine the moisture content of the cheese.

Six aluminum dishes with tops were cleaned and dried in an oven. They were then cooled in a dessicator, labelled and weighed accurately. To each dish was added 10.00 g of

cheese. The dishes were covered and placed in an oven heated to 100C and allowed to remain there for 24 hours. After removal they were placed in a dessicator to cool and re-weighed. The loss in weight times 10 gave the percentage moisture. These determinations were done in triplicate.

Fat content of cheese

The Pennsylvania method was used to determine the fat content of the cheese (Laboratory Manual,Milk Industry Foundation 1959).

4.5 g of cheese were placed into 50% cream test bottles. To each bottle was added 10 ml of water and the bottles placed in a water bath at 150F(66C). The bottles were shaken intermittently to disperse the sample and allowed to remain in the water bath for 10 minutes. Following this 17.6 ml of sulphuric acid (S.G. 1.82) was added slowly to each bottle. The bottles were centrifuged in a heated Cherry-Burrell centrifuge for five minutes. To each bottle was added enough hot water to,raise the level of the fat. The samples were recentrifuged for five minutes after which a few drops of glymol were added to facilitate easier reading of the fat column. The reading obtained times 2 gave the fat percentage. These determinations were done in triplicate.

Brine concentration of cheese

The brine concentration was calculated theoretically from the moisture and salt contents using the following formula (Foster *et al*.1961)

$$\left(\frac{\%\ \text{salt added}}{\%\ \text{moisture}}\right) \times 100$$

Growth of S. aureus Cas 243 in Trypticase Soy Broth

In an effort to determine the growth and behaviour of *S. aureus* Cas 243 in broth of varying pH and at different temperatures of incubation experiments were carried out using Trypticase Soy Broth (TSB) and two fermentors (Bio-Kulture Assembly,Fermantation Design Inc.,Penna.,U.S.A.) (Plate IV).

0.5 ml of a 24-hour culture of *S. aureus* Cas 243 was inoculated into each of two fermentors containing 3000 ml of TSB adjusted to the desired pH. The cultures were incubated for a minimum of 72 hours at the desired temperature. Bacterial enumerations were made using SPCA as the plating medium. Growth was also measured turbidimetrically at 600 mu using a Spectronic 20 spectrophotometer (Bausch and Lomb).

Experiments were carried out using pH 4.0, 5.0 and 6.0 at incubation temperatures of 12, 25 and 37C. In some of the runs the pH was kept constant by the addition, at intervals, of 6N NaOH. Samples of broth were kept to determine the production of enterotoxin.

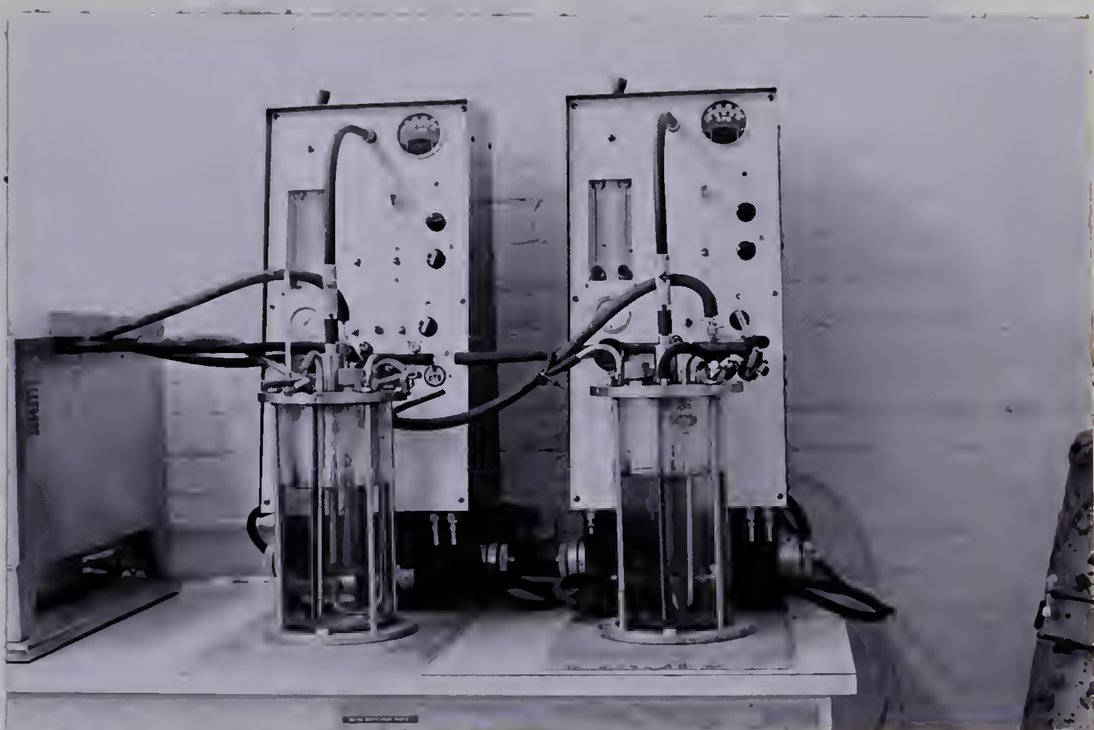


Plate IV. Fermentors used to grow S. aureus Cas 243
in Trypticase Soy Broth.

RESULTS

Manufacturing record of cheese

Data recorded during the manufacture of the various cheeses are shown in Tables 2-7. Changes in pH and titratable acidity followed a similar pattern to that illustrated in Fig.2

Total bacterial numbers of cheese during ripening

The changes in bacterial numbers occurring in the cheese during ripening over a period of 35-38 weeks are shown in Table 8 and Fig.4. It can be seen that all curves except one show an increase of numbers during the early stages of ripening. At a later stage there is a gradual decrease in numbers until a certain point when an increase in numbers appears. There appears to be a similar pattern in all the curves.

Numbers of viable S. aureus Cas 243 in cheese

The number of viable *S. aureus* Cas 243 occurring in the various cheeses during storage is shown in Table 9 and Fig.5. Except for cheeses A and E there is a gradual decrease of *S. aureus* throughout the ripening period. In cheeses A and E there is an increase of staphylococci during the first seven and 16 weeks respectively before noting a decrease in numbers.

Change of pH of cheese during storage

The change in pH of cheese during storage is shown in

Table 10 and illustrated graphically for cheese F in Fig.3. All of the other cheeses showed a similar trend.

Table 10. Change in pH during storage of cheese

Time (weeks)	Cheese A	B	D	E	F
0	5.30	5.30	5.35	5.40	5.40
4	-	-	-	5.36	5.35
6	-	-	-	5.35	-
7	5.25	-	5.27	-	-
9	5.25	-	5.26	-	-
10	-	5.23	-	-	5.28
12	-	-	-	5.30	5.27
13	5.21	-	-	-	-
14	-	-	-	5.28	5.24
15	5.20	5.19	5.24	-	-
16	-	-	-	-	5.23
17	-	5.18	-	5.26	-
18	5.19	-	-	-	-
19	-	5.16	5.21	-	5.21
20	5.15	-	-	5.23	-
22	5.15	5.14	5.20	5.21	5.20
24	5.13	5.12	5.18	5.18	5.18
27	5.13	5.11	5.15	5.15	5.19
30	-	-	5.15	5.11	5.12
32	5.11	5.10	-	-	5.11
33	-	-	5.13	5.08	-
34	-	5.08	-	-	-

Table 10 (Cont'd)

Time (weeks)	Cheese A	B	D	E	F
35	5.09	5.13	-	5.06	5.08
38	-	-	5.13	-	-

Coagulase activity

Coagulase activity of *S. aureus* Cas 243 isolated from the various cheeses was determined by the ability of cells grown in brain heart infusion broth to coagulate plasma. It was noted that the activity of the organism was the same as before inoculation into the cheese milk. All cultures coagulated the plasma in less than one hour.

Moisture, fat and brine concentration of cheese

The moisture, fat and brine concentration contents of the cheese after 40 weeks of storage is shown in Table 11. The results from these determinations were normal and within the range expected of Cheddar cheese.

Table 11. Moisture,fat and brine concentration of cheese

Cheese	%Moisture	% Fat	Brine conc.
A	37.14	33.20	4.04
B	36.08	32.80	4.15
C	35.60	32.00	4.08
D	36.68	32.00	4.08
E	35.84	31.20	4.18
F	38.07	32.00	3.94

Table 2. Manufacturing data for cheese A (S. aureus added together with starter)

Milk:- 580 lbs   Titratable acidity - 0.16%

Total bacterial count - 28×10^3 per ml   pH - 6.8

Coagulase-positive S. aureus - negative

Cheese:- Quantity of starter used - 8.7 lbs

Titratable acidity of starter - 0.70%

Titratable acidity of starter and milk - 0.17%

Quantity of rennet added - 48.0 ml

Total bacterial count in cheese milk - 1.76×10^7 per ml

S. aureus count in cheese milk - 1.8×10^4 per ml

S. aureus count in expelled whey - 8.1×10^4 per ml

S. aureus count in curd after hooping - 2.25×10^5 per g

Total bacterial count in curd after hooping - 1.25×10^7 per g

Amount of salt added - 1.2 lbs

Yield of cheese - 57 lbs   Cheese stored - 41.5 lbs

Changes in pH and titratable acidity during process

	pH	Titratable acidity(%)
At start of process	6.8	0.16
At time of cutting the curd	6.75	0.14
At time dipped	6.75	0.175
At time curd piled	6.10	0.25
At time of first turning	5.90	0.37
At time of milling	5.35	0.75

Time from the start of the process to milling - 285 mins.

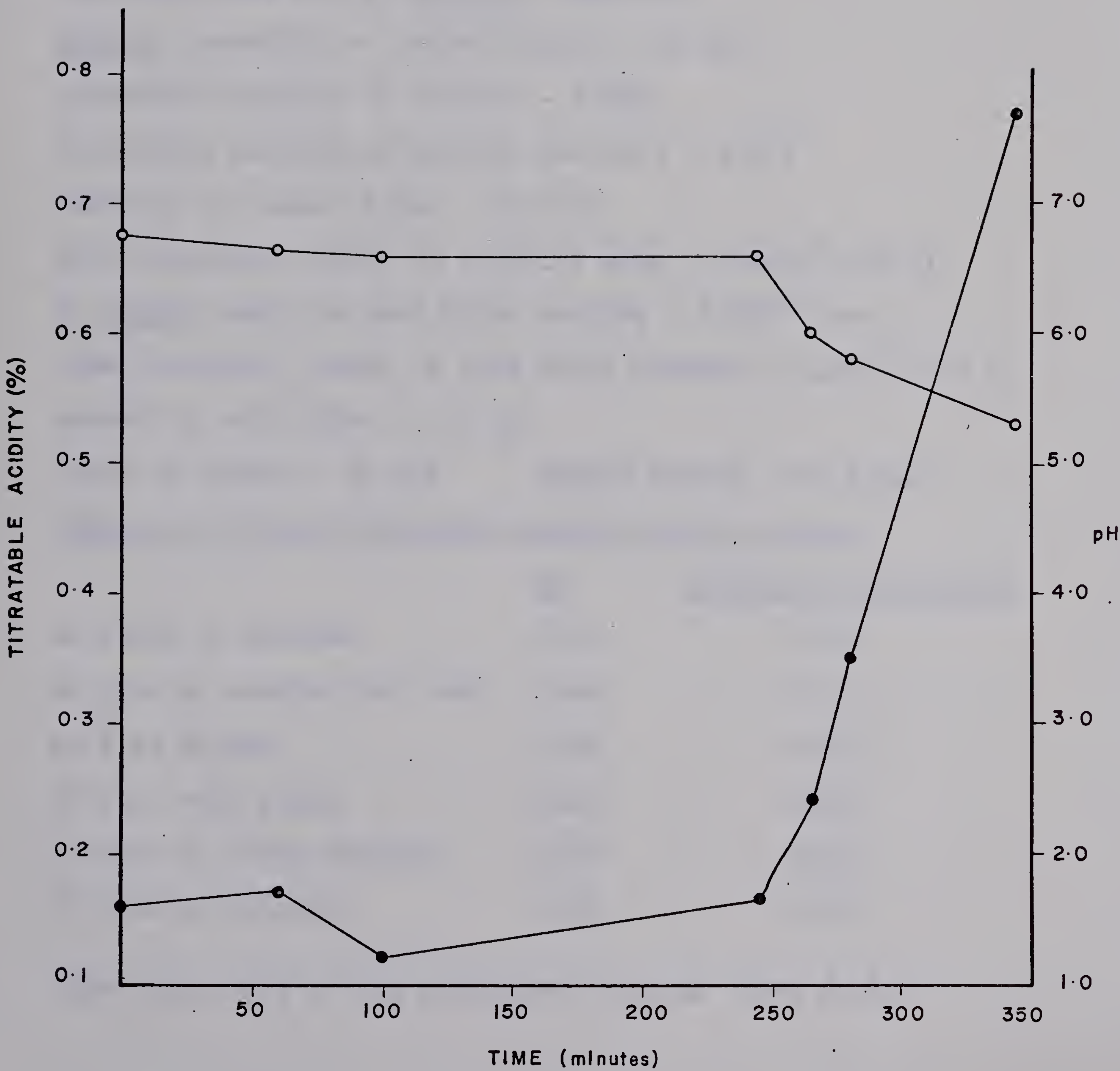


Fig. 2 Changes in pH and titratable acidity during manufacture of cheese (cheese B)

Table 3. Manufacturing data for cheese B (S. aureus added at salting)

Milk:- 510 lbs Titratable acidity - 0.16%

Total bacterial count - 8.1×10^3 per ml pH - 6.75

Coagulase-positive S. aureus - negative

Cheese:- Quantity of starter used - 7.5 lbs

Titratable acidity of starter - 0.75%

Titratable acidity of starter and milk - 0.17%

Quantity of rennet added - 43.0 ml

Total bacterial count in expelled whey - 1.9×10^7 per ml

S. aureus count in curd after hooping - 9.7×10^5 per g

Total bacterial count in curd after hooping - 3.2×10^6 per g

Amount of salt added - 1.1 lbs

Yield of cheese - 50 lbs Cheese stored - 41.0 lbs

Changes in pH and titratable acidity during process

	pH	Titratable acidity(%)
At start of process	6.75	0.16
At time of cutting the curd	6.60	0.12
At time dipped	6.60	0.165
At time curd piled	6.05	0.25
At time of first turning	5.80	0.35
At time of milling	5.30	0.77

Time from start of the process to milling - 285 mins.

Table 4. Manufacturing data for cheese C (S. aureus not added - control)

Milk:- 510 lbs Titratable acidity - 0.16%

Total bacterial count - 27.5×10^3 per ml pH - 6.75

Coagulase-positive S. aureus - negative

Cheese:- Quantity of starter used - 7.5 lbs

Titratable acidity of starter - 0.75%

Titratable acidity of starter and milk - 0.175%

Quantity of rennet added - 43.0 ml

Total bacterial count in expelled whey - 38×10^5 per ml

S. aureus count in curd after hooping - negative

Total bacterial count in curd after hooping - 115×10^7 per g

Amount of salt added - 1.1 lbs

Yield of cheese - 50 lbs Cheese stored - 41.5 lbs

Changes in pH and titratable acidity during process

	pH	Titratable acidity(%)
At start of process	6.75	0.16
At time of cutting the curd	6.50	0.13
At time dipped	6.50	0.16
At time curd piled	6.00	0.22
At time of first turning	5.85	0.35
At time of milling	5.30	0.76

Time from the start of the process to milling - 270 mins.

Table 5. Manufacturing data for cheese D (S. aureus added at rennetting)

Milk:- 450 lbs Titratable acidity - 0.16%

Total bacterial count - 5.2×10^3 per ml pH - 6.8

Coagulase-positive S. aureus - negative

Cheese:- Quantity of starter used - 6.75 lbs

Titratable acidity of starter - 0.77%

Titratable acidity of starter and milk - 0.17%

Quantity of rennet added - 39.0 ml

S. aureus count in cheese milk - 2.0×10^6 per ml

S. aureus count in expelled whey - 7.7×10^4 per ml

Total bacterial count in expelled whey - 8.4×10^5 per ml

S. aureus count in curd after hooping - 4.2×10^7 per g

Total bacterial count in curd after hooping - 1.3×10^9 per g

Amount of salt added - 15 ounces

Yield of cheese - 44 lbs Cheese stored - 40.5 lbs

Changes in pH and titratable acidity during process

	pH	Titratable acidity(%)
At start of process	6.80	0.16
At time of cutting the curd	6.70	0.12
At time dipped	6.70	0.165
At time curd piled	6.15	0.24
At time of first turning	5.90	0.35
At time of milling	5.35	0.75

Time from the start of the process to milling - 285 mins.

Table 6. Manufacturing data of cheese E (S. aureus added at start of cooking)

Milk:- 475 lbs Titratable acidity - 0.15%

Total bacterial count - $22.5x10^{3}$ per ml pH - 6.75

Coagulase-positive S. aureus - negative

Cheese:- Quantity of starter used - 7.0 lbs

Titratable acidity of starter - 0.75%

Titratable acidity of starter and milk - 0.165%

Quantity of rennet added - 41.0 ml

S. aureus count at start of cooking - $1.7x10^{5}$ per ml

Total bacterial count at start of cooking - $22x10^{6}$ per ml

S. aureus count in expelled whey - $2.5x10^{5}$ per ml

Total bacterial count in expelled whey - $6.5x10^{8}$ per ml

S. aureus count in curd after hooping - $1.4x10^{6}$ per g

Total bacterial count in curd after hooping - $1.2x10^{9}$ per g

Amount of salt added - 1 lb

Yield of cheese - 47 lbs Cheese stored - 41.0 lbs

Changes in pH and titratable acidity during process

	pH	Titratable acidity(%)
At start of process	6.75	0.15
At time of cutting the curd	6.70	0.12
At time dipped	6.70	0.16
At time curd piled	6.20	0.23
At time of first turning	6.00	0.35
At time of milling	5.40	0.76

Time recorded from start of the process to milling -285 mins.

Table 7. Manufacturing data for cheese F (S. aureus added 12 hours before starter)

Milk:- 500 lbs Titratable acidity - 0.16%

Total bacterial count - 2.15×10^3 per ml pH - 6.85

Coagulase-positive S. aureus - negative

S. aureus added - 1.25×10^6 per ml Storage temperature - 58F

S. aureus count after 12 hours storage - 8.0×10^5 per ml

Cheese:- Quantity of starter used - 7.5 lbs

Titratable acidity of starter - 0.78%

Titratable acidity of starter and milk - 0.175%

Quantity of rennet added - 43.0 ml

S. aureus count in expelled whey - 7.3×10^6 per ml

Total bacterial count in expelled whey - 1.09×10^9 per ml

S. aureus count in curd after hooping - 2.8×10^8 per g

Total bacterial count in curd after hooping - 1.1×10^9 per g

Amount of salt added - 1.2 lbs

Yield of cheese - 49 lbs Cheese stored - 40.5 lbs

Changes in pH and titratable acidity during process

	pH	Titratable acidity(%)
At start of process	6.82	0.165
At time of cutting the curd	6.80	0.13
At time dipped	6.80	0.17
At time curd piled	6.25	0.24
At time of first turning	6.05	0.37
At time of milling	5.40	0.75

Time from the start of the process to milling - 290 mins.

Table 8. Total bacterial changes in cheese during ripening

Time (weeks)	Viable count of bacteria per gram in cheese:-				
	A	B	D	E	F
0	125×10^5	32×10^5	130×10^7	120×10^6	110×10^7
4	-	-	-	150×10^6	140×10^7
6	-	-	-	210×10^6	-
7	208×10^5	-	85×10^7	-	-
9	-	61×10^7	68×10^7	-	-
10	-	-	-	-	97×10^8
12	-	-	-	49×10^9	124×10^8
13	95×10^5	-	33×10^7	-	-
14	-	-	-	83×10^9	208×10^8
15	64×10^5	110×10^7	-	-	-
16	-	-	-	-	46×10^9
18	28×10^5	-	-	-	-
19	-	69×10^7	-	-	25×10^9
20	20×10^5	-	101×10^5	120×10^8	-
21	-	39×10^7	-	-	-
22	-	-	146×10^5	80×10^8	21×10^9
23	-	21×10^7	-	-	-
24	120×10^4	-	190×10^5	51×10^8	180×10^8
27	84×10^4	195×10^5	251×10^5	26×10^8	150×10^8
29	14×10^4	56×10^6	-	-	-
30	-	-	89×10^6	45×10^8	90×10^8
31	-	20×10^6	-	-	-
33	-	-	24×10^7	68×10^8	-

Table 8. (cont'd)

Time (weeks)	Viable count of bacteria per gram in cheese:-				
	A	B	D	E	F
34	-	$93x10^6$	-	-	-
35	$120x10^4$	-	$31x10^7$	$88x10^8$	$49x10^8$
38	-	$170x10^6$	-	-	-

Table 9. Numbers of S. aureus Cas 243 in cheese during ripening

Time (weeks)	Viable count of S. aureus per g in cheese:-				
	A	B	D	E	F
0	$225x10^3$	$97x10^4$	$42x10^6$	$14x10^5$	$28x10^7$
4	-	-	-	$66x10^5$	$13x10^7$
6	-	-	-	$90x10^5$	-
7	$68x10^4$	-	$110x10^5$	-	-
9	$51x10^5$	$75x10^4$	$81x10^5$	-	-
10	-	-	-	-	$81x10^6$
12	$180x10^3$	-	-	$64x10^6$	$70x10^6$
13	-	-	$20x10^5$	-	-
14	-	-	-	$120x10^6$	$66x10^6$
15	$95x10^3$	$34x10^4$	-	-	-
16	-	-	-	-	$50x10^6$
18	$54x10^3$	-	-	-	-
19	-	$11x10^4$	$14x10^5$	-	$20x10^6$
20	$41x10^3$	-	-	$61x10^6$	-
21	-	$98x10^3$	-	-.	-
22	-	-	$83x10^4$	$100x10^5$	$81x10^5$

Table 9. (cont'd)

Time (weeks)	Viable count of *S. aureus* per g in cheese:- A	B	D	E	F
23	-	$79x10^3$	-	-	-
24	$75x10^2$	-	$65x10^4$	$60x10^5$	$72x10^5$
27	$62x10^2$	$50x10^3$	$180x10^3$	$32x10^5$	$50x10^5$
29	$56x10^2$	$26x10^3$	-	-	-
30	-	-	$72x10^3$	$100x10^4$	$45x10^5$
31	-	$16x10^3$	-	-	-
32	$50x10^2$	$81x10^2$	-	-	$38x10^5$
33	-	-	$20x10^3$	$91x10^4$	-
35	$49x10^2$	-	$80x10^2$	$80x10^4$	$28x10^5$
38	-	$41x10^2$	-	-	-

Toxin production in cheese

The ability of the organism to produce toxin in the various cheeses was expressed by the width of the migrating antigen-antibody precipitate band after 24 to 72 hours of incubation. Examination of all cheeses prepared showed that there was no detectable enterotoxin in any of the samples. The age of the cheese examined varied from 24 to 28 weeks.

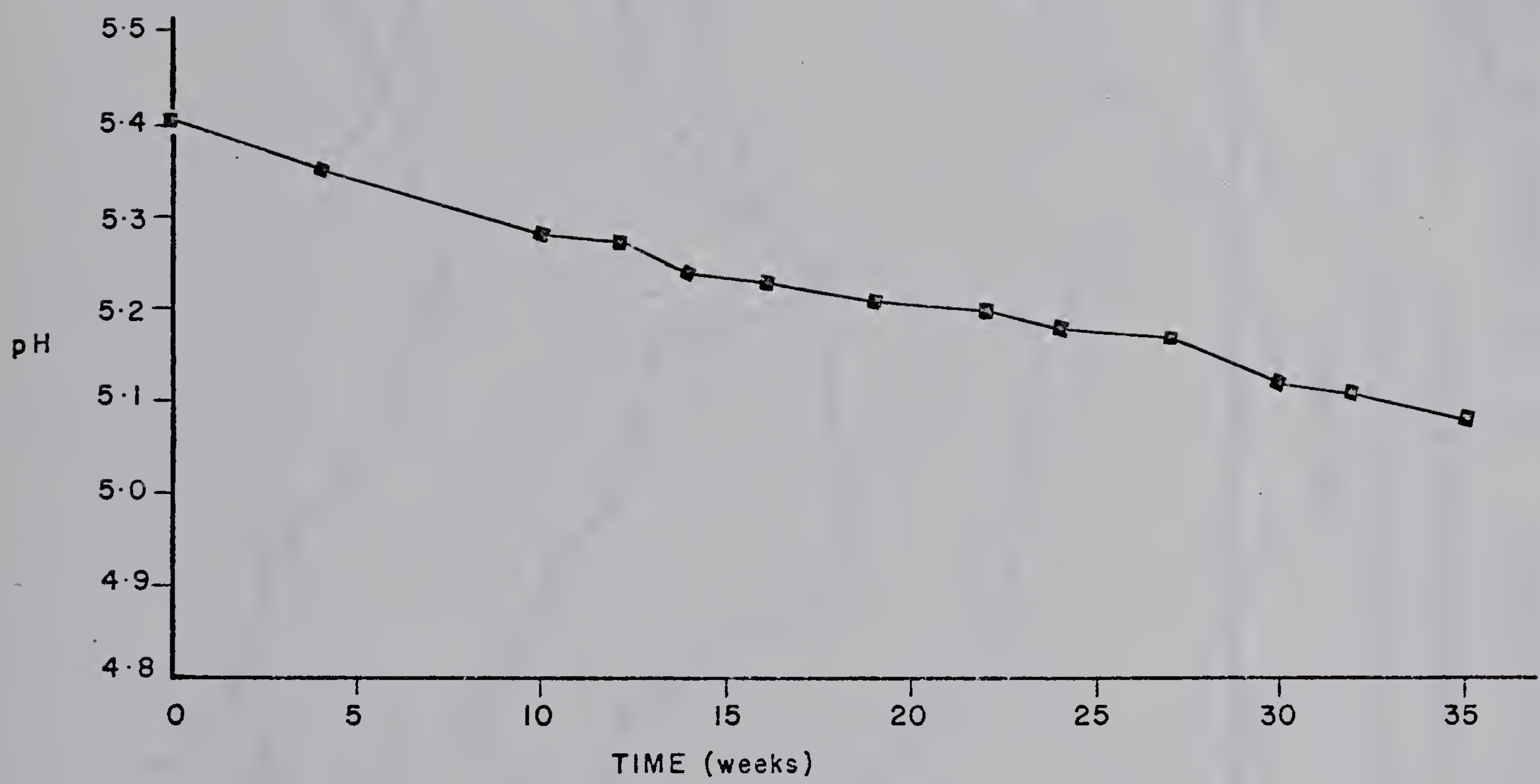


Fig. 3 pH change during storage of cheese (cheese F).

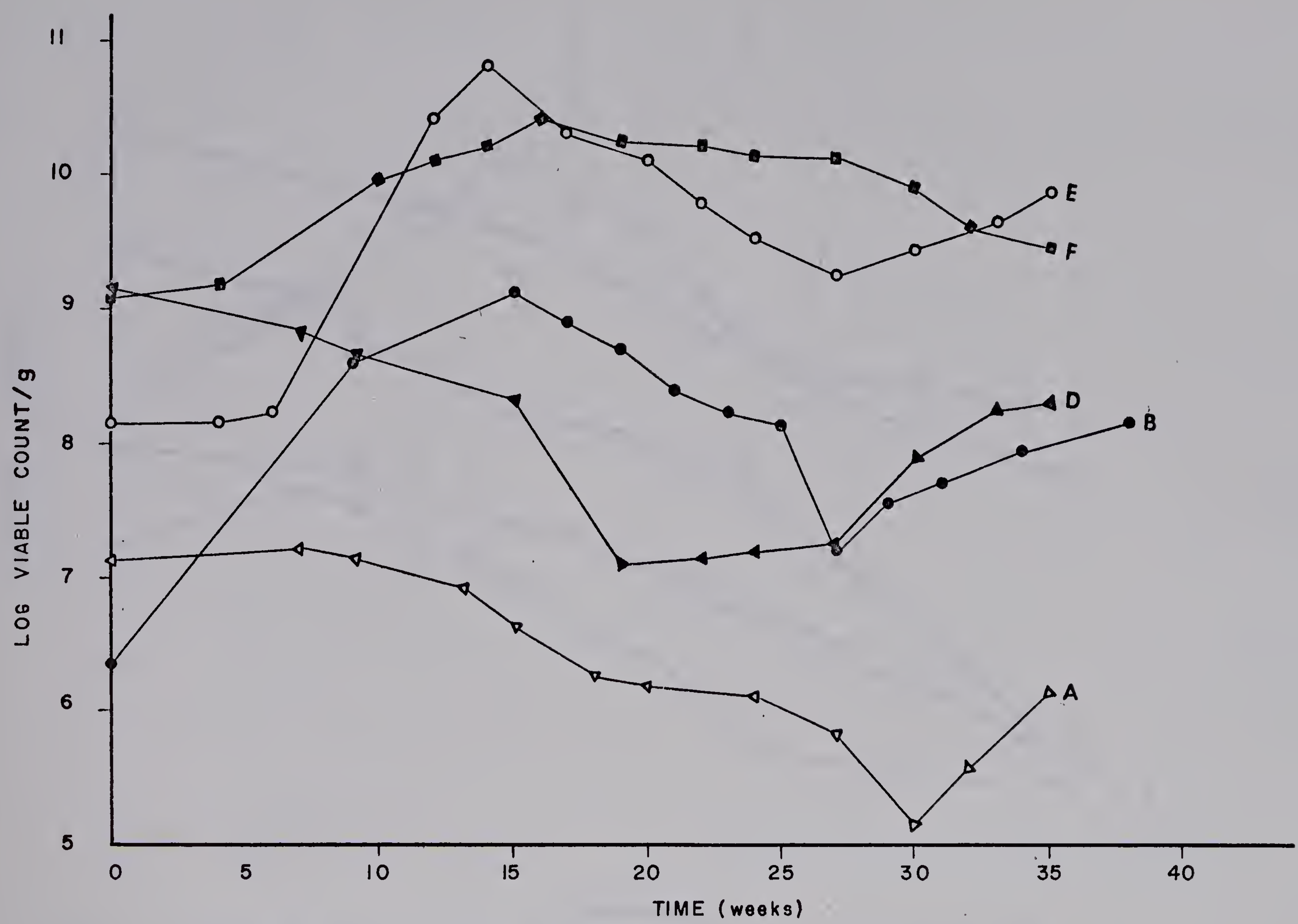


Fig. 4. Total bacterial count of cheese during storage at 50F.

A - *S. aureus* added together with starter

F - *S. aureus* added 12 hours before starter

B - *S. aureus* added at salting

E - *S. aureus* at start of cooking

D - *S. aureus* added at rennetting

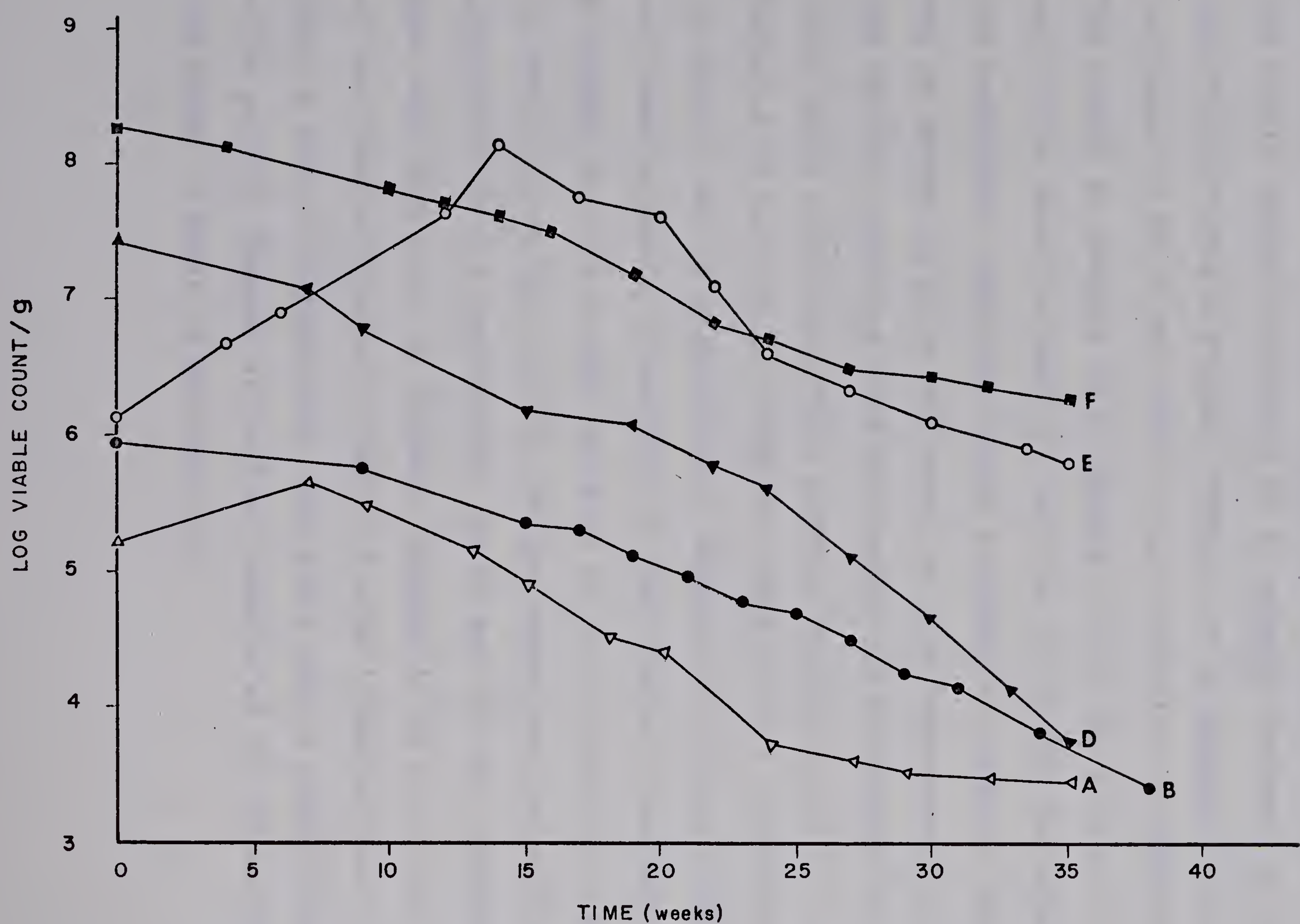
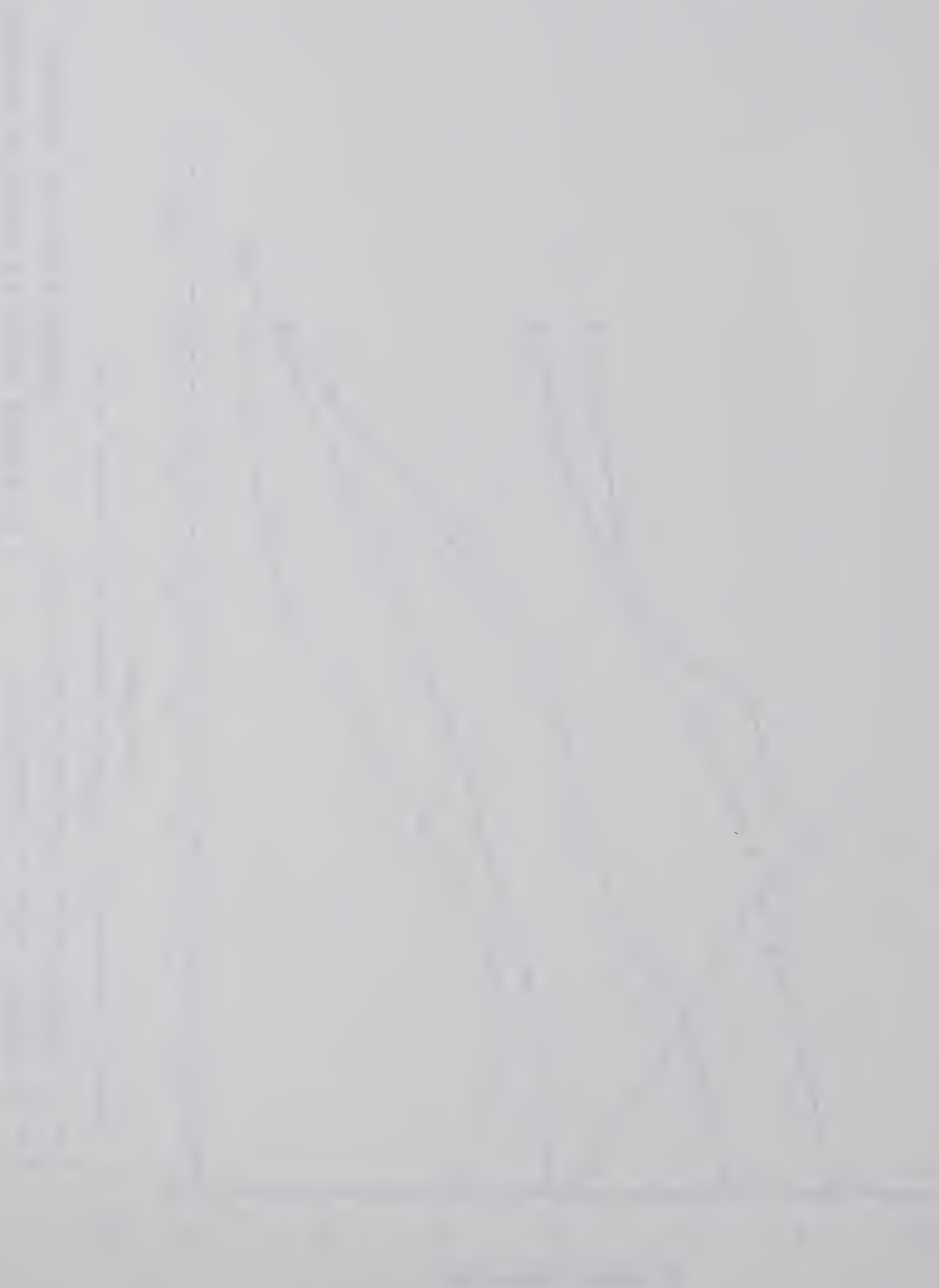


Fig. 5 Change in numbers of S. aureus in cheese during storage at 50F.

A - S. aureus added together with starter B - S. aureus added at salting

D - S. aureus added at rennetting E - S. aureus added at start of cooking

F - S. aureus added 12 hours before starter



Growth of *S. aureus* Cas 243 in Trypticase Soy Broth

The change in numbers of *S. aureus* in TSB (pH 4.0) at 12, 25 and 37C is shown in Table 12 and Fig. 6. At 12C there was little change in numbers of *S. aureus*, however, at 25 and 37C there was a gradual decrease of *S. aureus*. There was no change in pH of the broth at all incubation temperatures. The change in numbers of *S. aureus* in TSB (pH 5.0) at 12, 25 and 37C is shown in Table 13 and Fig. 7. At 12C the organisms are contained remaining fairly constant in numbers. There was no change in pH of the broth at this temperature. At 25, and 37C the organisms grew well until the pH dropped to a level which restricted their growth. Changes in pH of the broth at 25 and 37C are shown in Table 15 with illustrations in Figs. 9 and 10 respectively. Changes in optical density are shown in Table 16 and Fig. 8. The change in numbers of *S. aureus* Cas 243 in TSB (pH 6.0) at 12, 25 and 37C is shown in Table 14 and Fig. 11. At 12C the organisms grew well after adjustment to the temperature. At 25 and 37C the organisms grew well until the pH dropped. Changes in pH at 25 and 37C of the broth are shown in Table 15 with illustrations in Figs. 9 and 10 respectively. There was only a slight change of pH at 12C. Changes in optical density at 12, 25 and 37C are shown in Table 17 and Fig. 12.

Table 12 Growth of S. aureus in TSB (pH 4.0) at 12,25 and 37C

Hours of incubation	Viable count of S. aureus($\times 10^3$) per ml at incubation temperature of:-		
	12C	25C	37C
0	136	15	79
4	116	-	-
8	96	11.5	53
16	100	10.1	39
24	125	9.1	28
32	130	8.8	20
40	130	8.5	21
48	106	6.9	13.5
56	96	6.5	8.4
64	104	6.6	5.6
72	125	4.9	5.1
80	100	3.3	4.6
92	-	-	3.9
96	98	2.0	-

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Table 13. Growth of S. aureus in TSB (pH 5.0) at 12,25 and 37C

Hours of incubation	Viable count of S. aureus($\times 10^4$) per ml at incubation temperature of:-		
	12C	25C	37C
0	3.7	3.6	24
4	-	3.7	36
8	1.25	8.6	91
16	6.4	35.6	18,000
20	-	150.0	-
24	7.8	413.0	6,000
28	-	1800.0	-
32	6.6	4800.0	800
40	3.9	26,000	430
48	8.2	16,000	250
56	6.8	-	280
64	7.7	24,000	-
72	9.8	12,000	170
80	7.5	-	-
88	8.4	-	-
96	8.1	-	-

Table 14. Growth of S. aureus in TSB (pH 6.0) at 12,25 and 37C

Hours of incubation	Viable count of S. aureus ($\times 10^4$) per ml at incubation temperature of:-		
	12C	25C	37C
0	5.2	4.4	36
4	-	11.6	400
8	6.8	150.0	26,000
16	8.2	18,700	68,000
20	-	39,000	-
24	7.9	74,000	18,000
32	9.0	74,000	7,000
40	16.0	45,000	3,400
48	75.0	50,000	1,950
56	134.0	-	1,600
64	260.0	18,000	-
72	370.0	9,700	1,400
80	2,500	-	-

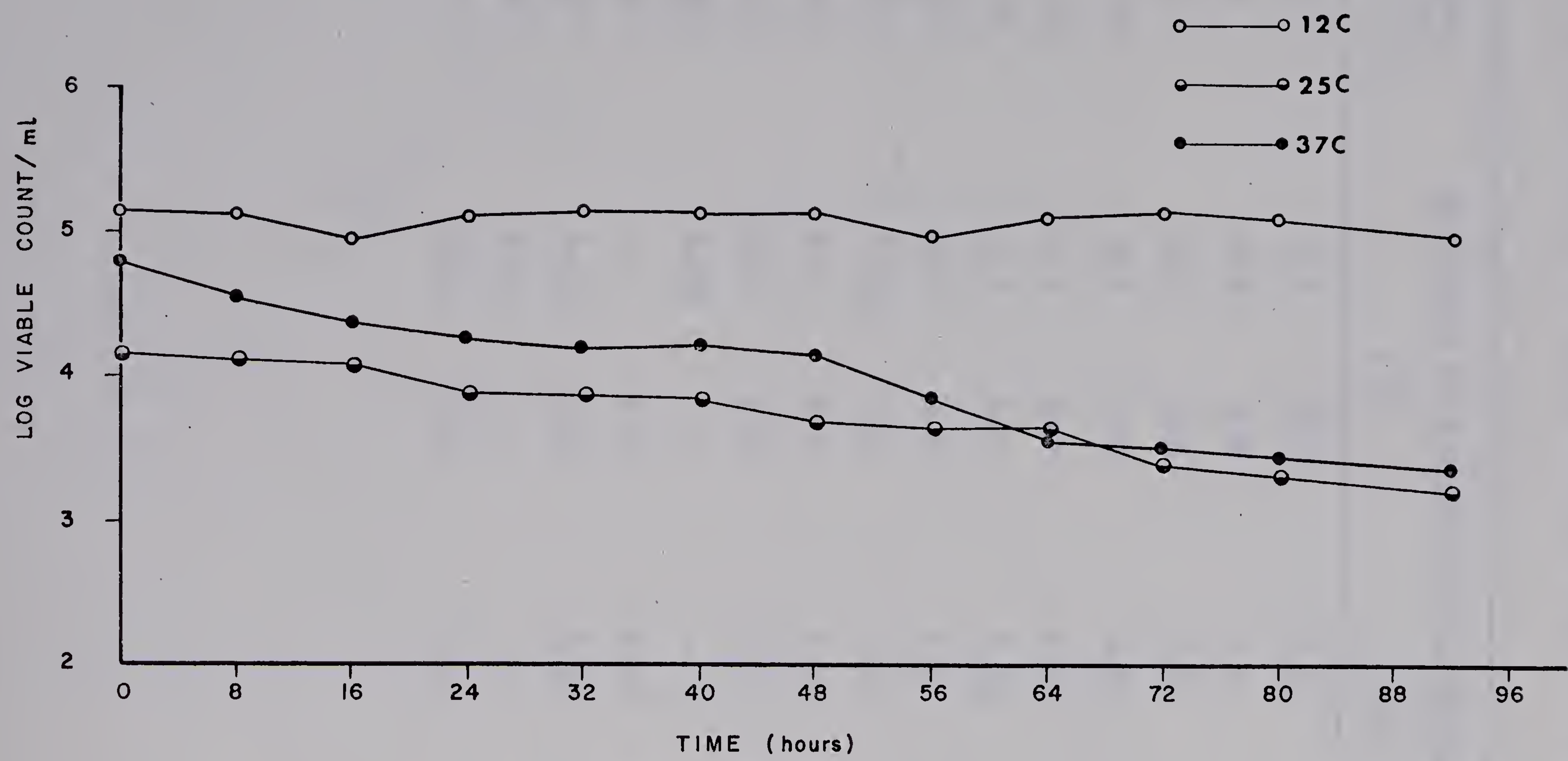

Fig. 6 Change in numbers of _S. aureus_ in TSB (pH 4.0) at 12,25 and 37C

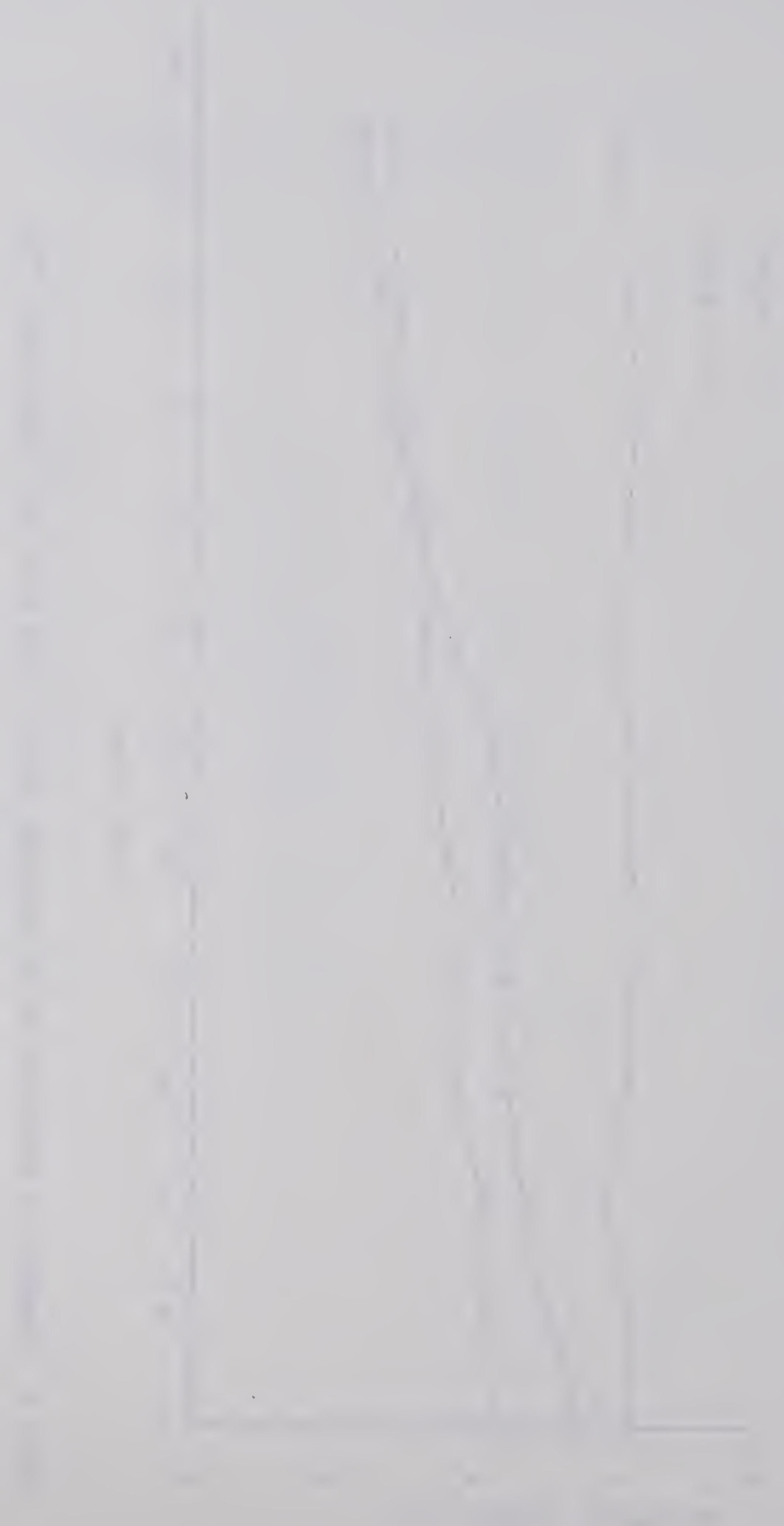

Table 15. Changes in pH of TSB (pH 5.0 & 6.0) at 25 and 37C

Time (hours)	pH change at incubation temperature of:-			
	25C		37C	
0	5.0	6.0	5.0	6.0
4	5.0	6.0	5.0	5.85
8	5.0	6.0	4.9	5.2
16	5.0	5.4	4.8	4.7
20	5.0	5.0	4.6	4.6
24	5.0	4.7	4.45	4.6
28	4.9	4.5	4.3	4.6
32	4.85	4.5	4.28	4.6
40	4.6	4.5	4.28	4.6
48	4.5	4.5	4.2	4.6
52	4.45	-	-	-
56	-	4.5	4.15	4.6
64	4.35	4.5	4.15	4.6
68	4.35	-	-	-
72	4.35	4.5	4.15	4.6

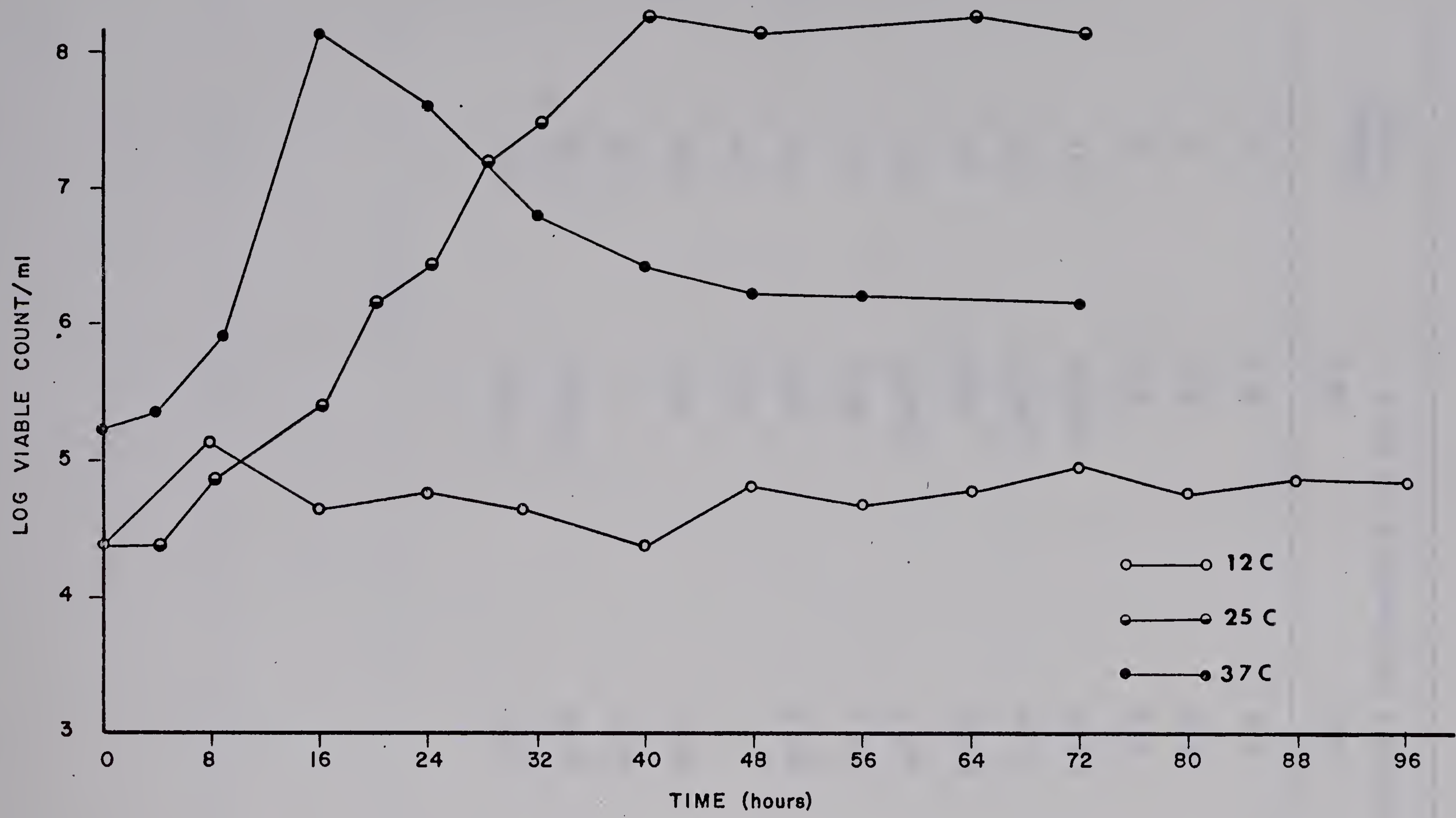

Fig. 7 Change in numbers of S. aureus in TSB (pH 5.0) at 12,25 and 37C

Table 16. Changes in O.D.(600 mu) of TSB (pH 5.0) at 25 and 37C

Time (hours)	O.D. at incubation temperature of:-	
	25C	37C
0	0.0	0.0
4	0.0	0.0
8	0.0	0.0
16	0.005	0.17
20	0.008	0.34
24	0.02	0.42
28	0.048	0.44
32	0.09	0.44
40	0.28	0.44
44	0.35	-
48	0.36	0.44
52	-	0.44
64	0.385	0.44
72	0.40	0.44

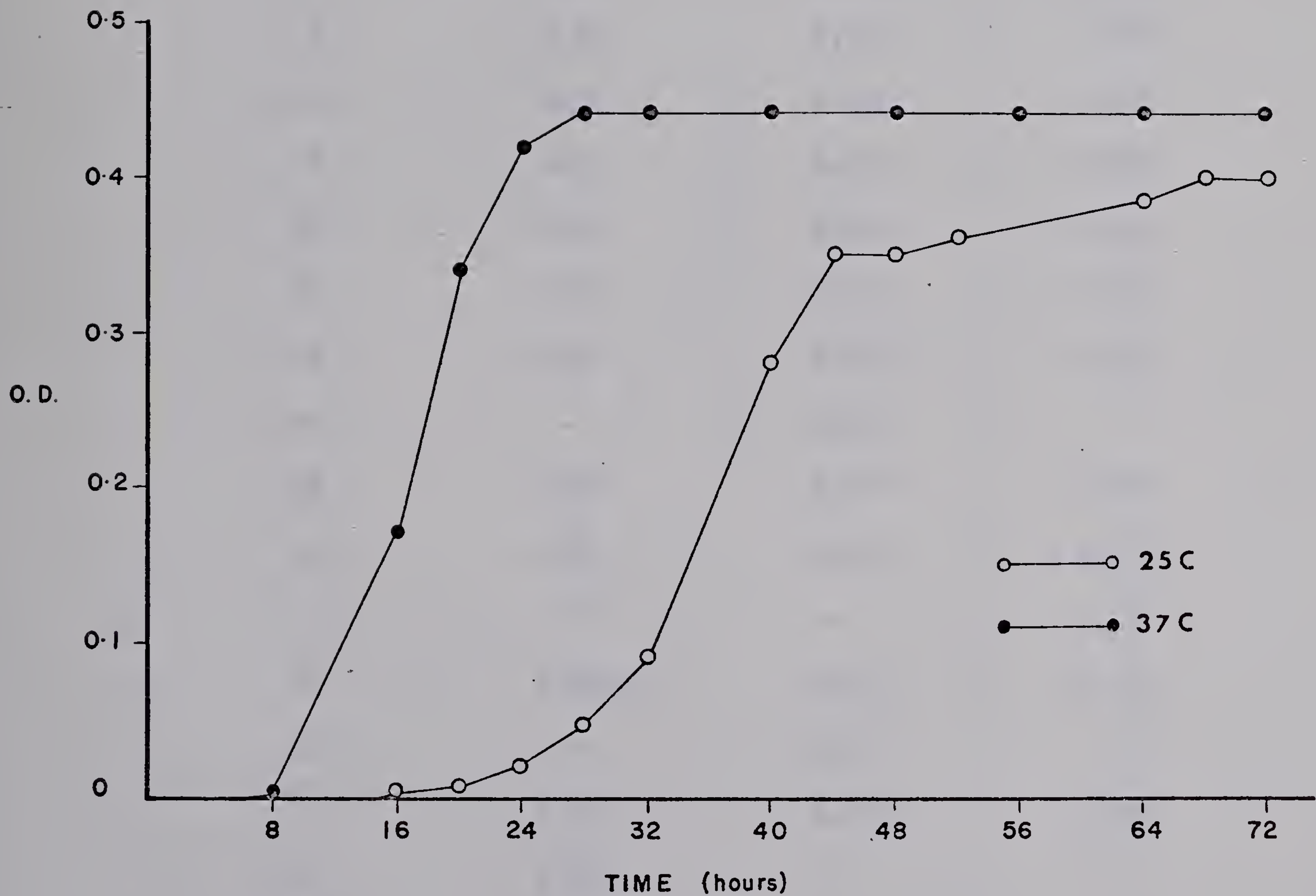

Fig. 8 Growth of S. aureus in TSB (pH 5.0) at 25 and 37C

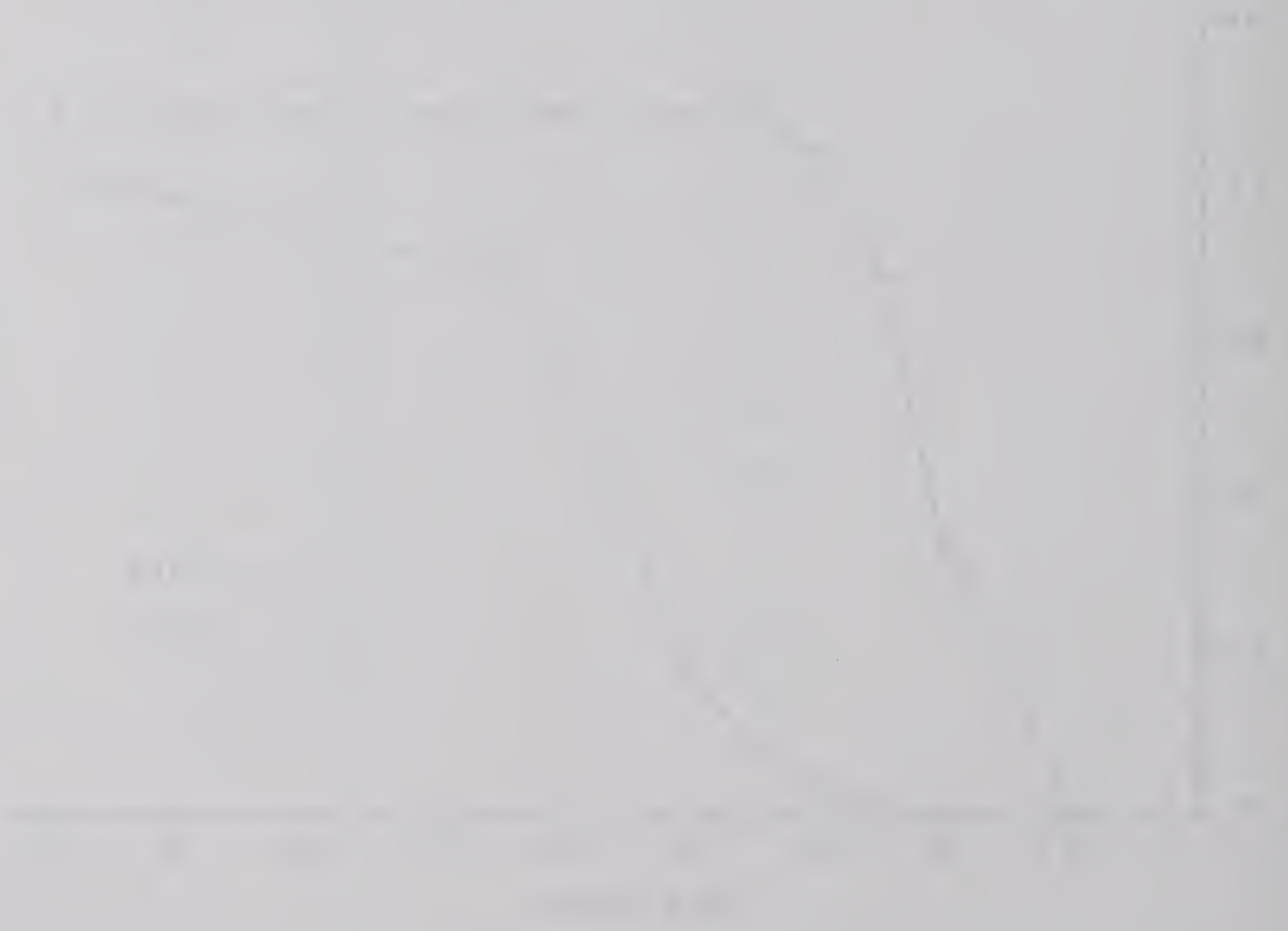

Table 17. Changes in O.D.(600 mu) of TSB (pH 6.0) at 12,25 and 37C

Time (hours)	O.D. at incubation temperature of:-		
	12C	25C	37C
0	0.0	0.0	0.0
4	0.0	0.01	0.05
8	0.0	0.02	0.48
16	0.0	0.28	0.68
20	0.0	0.50	0.70
24	0.0	0.62	0.70
28	0.0	0.65	0.70
32	0.0	0.66	0.70
40	0.0	0.66	0.70
44	-	0.64	-
48	0.0	0.64	0.70
52	0.0	0.65	0.70
56	-	-	0.70
64	0.006	0.66	0.70
68	-	0.66	-
72	0.01	0.66	0.70
80	0.02	-	-
88	0.15	-	-
96	0.35	-	-
104	0.58	-	-

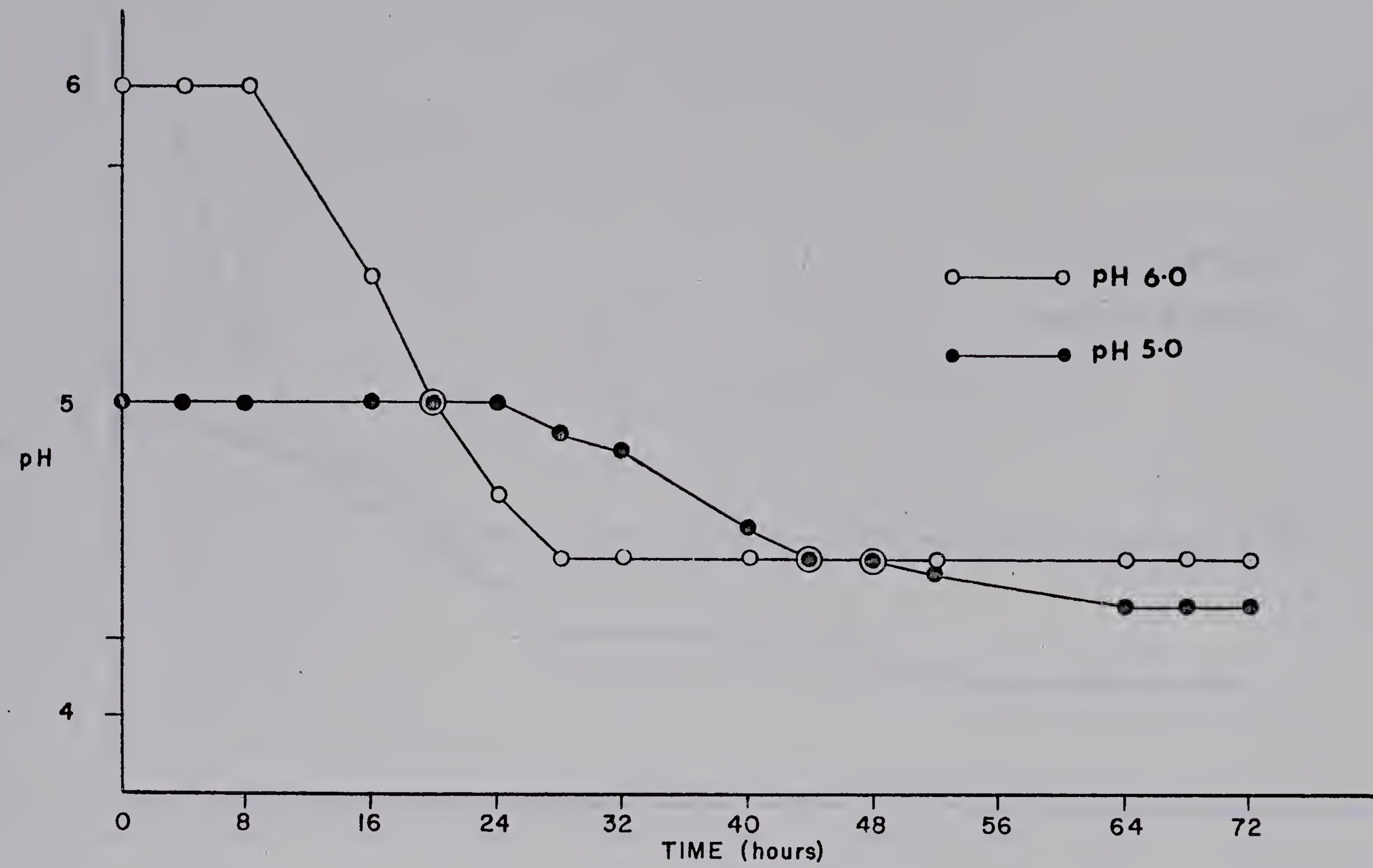

Fig. 9 Change in pH of TSB (pH 5.0 & 6.0) at 25C

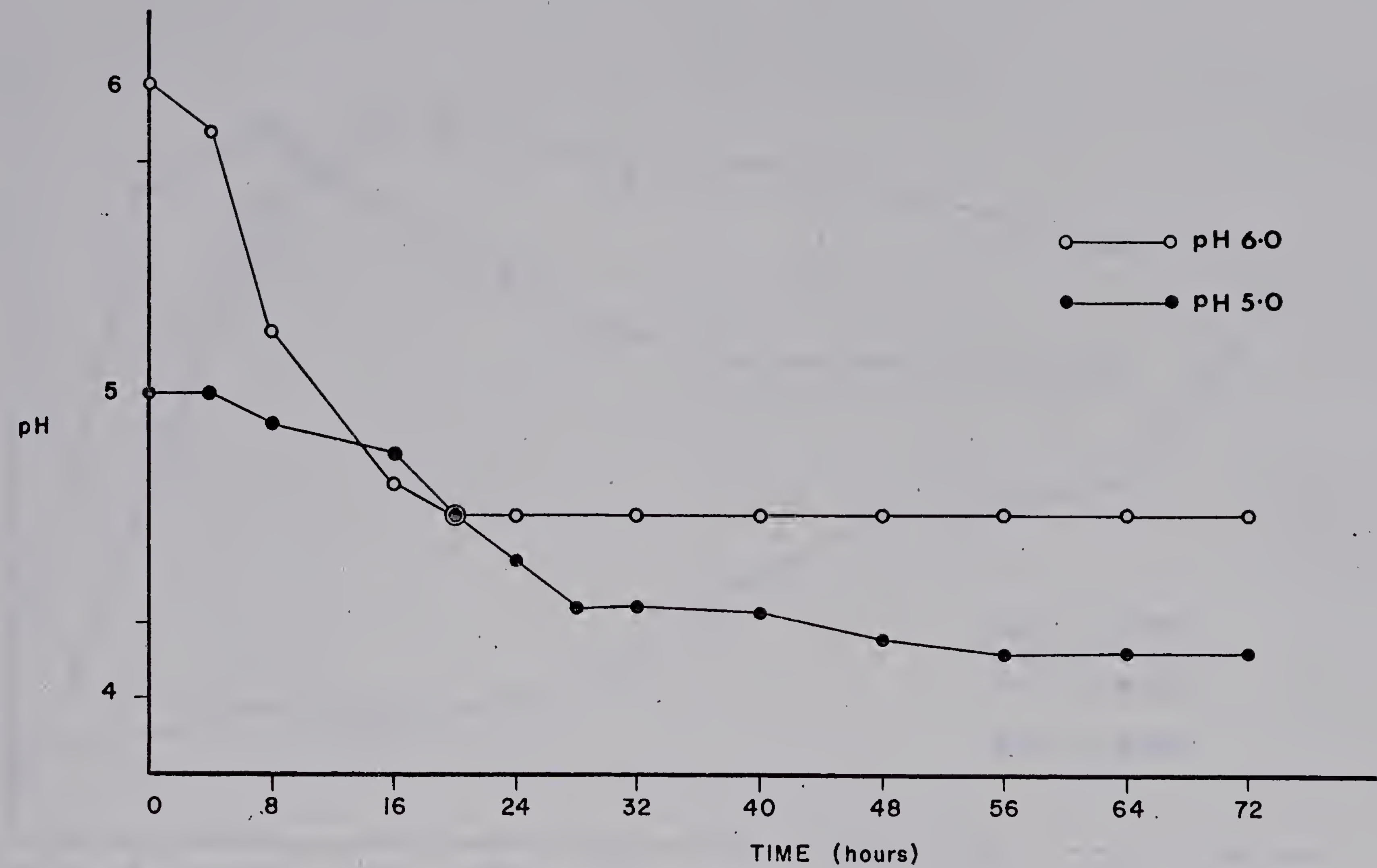

Fig. 10 Change in pH of TSB (pH 5.0 & 6.0) at 37C

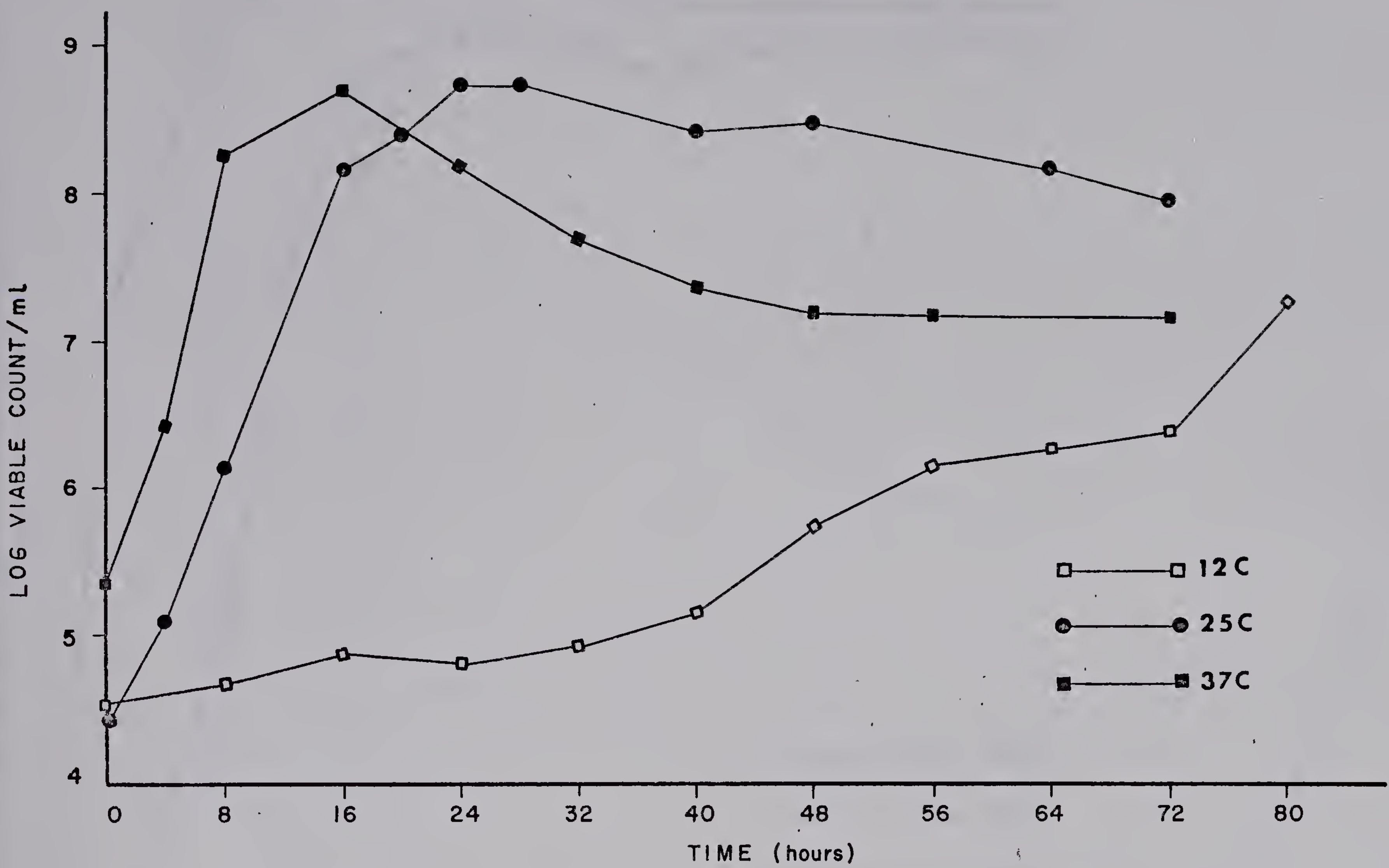

Fig. 11 Change in numbers of S. aureus in TSB (pH 6.0) at 12,25 and 37C

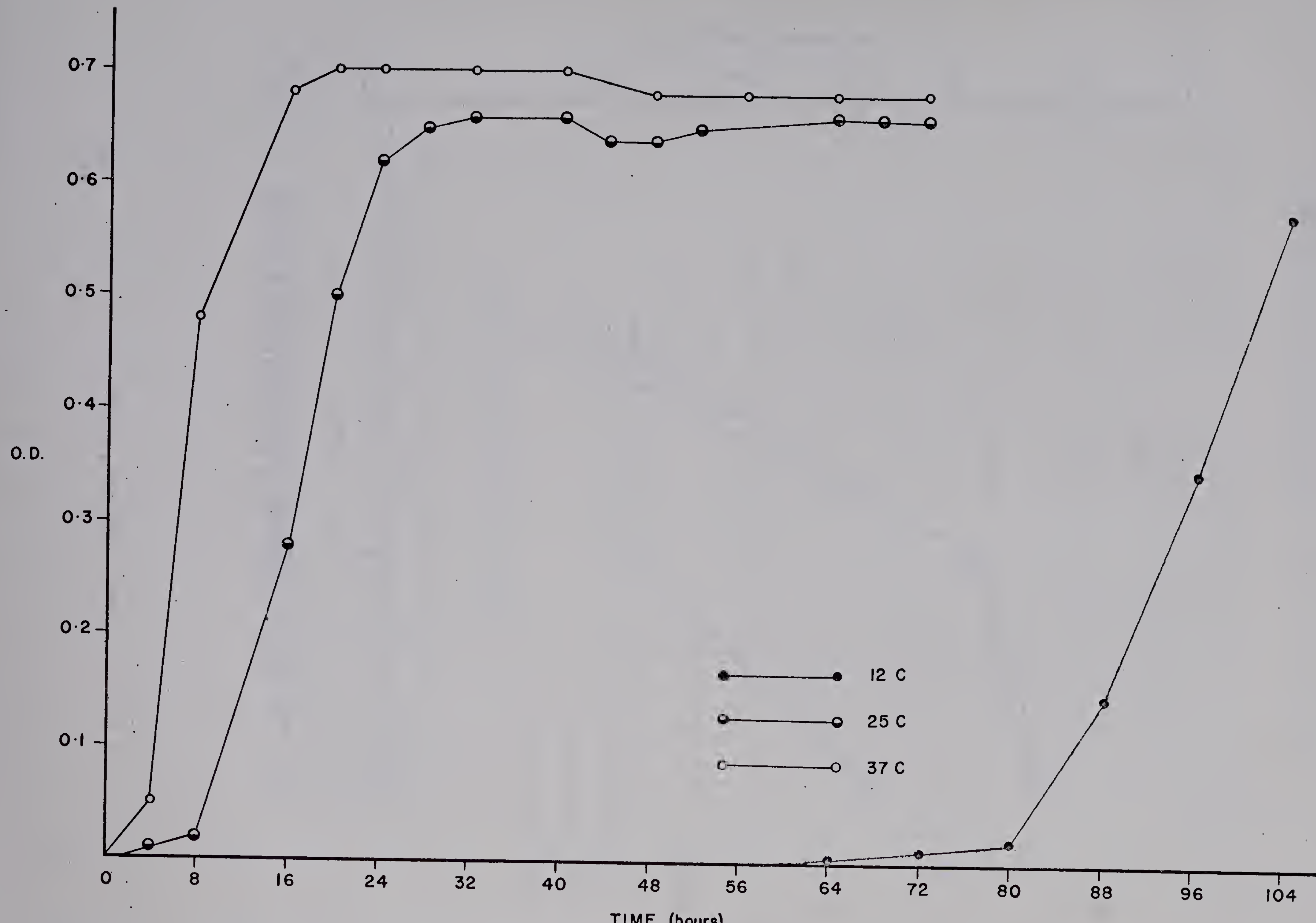

Fig. 12 Growth of _S. aureus_ in TSB (ph 6.0) at 12,25 and 37C

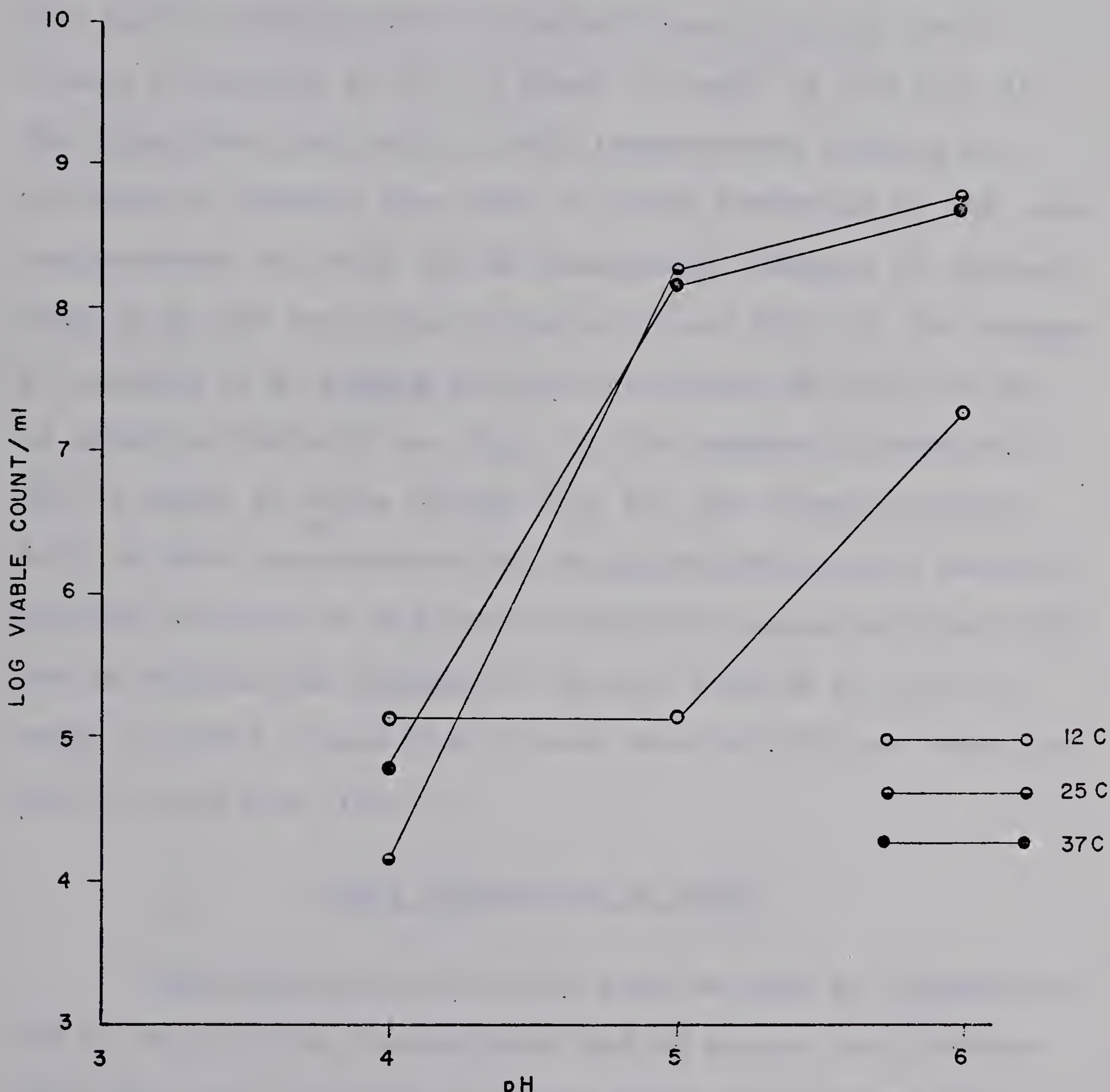

Fig. 13 Maximum numbers of S. aureus in TSB (pH 4.0, 5.0 & 6.0) at 12,25 and 37C

Growth of S. aureus Cas 243 in Trypticase Soy Broth of constant pH

The change in numbers of *S. aureus* in TSB of constant pH (5.0) at 25C is shown in Table 18 and Fig. 14. The change in numbers at 37C is shown in Table 18 and Fig. 16. The organisms grew well at both temperatures showing an increase of numbers over that of broth incubated at the same temperatures but with the pH unadjusted. Changes in optical density at 25C are shown in Table 19 and Fig. 17. The change in numbers of *S. aureus* in TSB of constant pH (6.0) at 25C is shown in Table 20 and Fig. 14. The change in numbers at 37C is shown in Table 20 and Fig. 16. The organisms grew well at both temperatures and surpassed the maximum numbers reached in broth of the same incubation temperature but with the pH unadjusted. Changes in optical density at 25C are shown in Table 21 and Fig. 15 and those at 37C are shown in Table 21 and Fig. 17.

Toxin production in broth

Examination of all broths used to grow *S. aureus* Cas 243 at the various temperatures and pH showed that enterotoxin was only detected in broths which had a pH of 6.0. The amount of toxin produced was not quantitated.

Table 18. Growth of S. aureus in TSB of constant pH (5.0) at 25 and 37C

Hours of incubation	Viable count of S. aureus($x10^4$) per ml at incubation temperature of:-	
	25C	37C
0	3.7	0.62
4	-	0.21
8	1.9	1.1
16	4.0	440.0
23	-	23,000
25	200.0	-
28	-	31,000
32	-	41,000
33	6,000	-
36	-	28,000
37	20,000	-
40	117,000	22,000
44	117,000	20,300
48	101,000	17,000

Table 19. Changes in O.D. of TSB of constant pH(5.0) at 25 and 37C

Time (hours)	Changes in O.D. at incubation temperature of:- 25C	37C
0	0.0	0.0
4	-	0.005
8	0.0	0.008
10	0.0	0.01
12	0.005	0.01
14	0.005	0.01
16	0.008	0.02
18	-	0.04
20	0.01	-
21	-	0.115
23	-	0.18
25	0.03	-
26	-	0.35
28	-	0.385
32	-	0.48
33	0.11	-
36	-	0.52
37	0.29	-
40	-	0.56
44	0.53	0.56
48	0.54	0.56
52	0.54	-
56	0.54	-

Table 20. Growth of S. aureus in TSB of constant pH(6.0) at 25 and 37C

Hours of incubation	Viable count of S. aureus($\times 10^4$) per ml at incubation temperature of:-	
	25C	37C
0	1.7	0.43
4	-	3.9
8	3.3	1,330
10	-	240,000
14	-	180,000
16	24,000	-
18	-	97,000
20	80,000	-
23	-	84,000
25	350,000	-
28	-	61,000
32	-	41,000
33	240,000	-
36	-	58,000
40	-	39,000
44	100,000	25,000
48	90,000	15,000

Table 21. Changes in O.D. of TSB of constant pH(6.0) at 25 and 37C

Time (hours)	Changes in O.D. at incubation temperature of:- 25C	37C
0	0.0	0.0
4	-	0.01
8	0.01	0.04
10	-	0.26
12	0.015	0.60
14	-	0.86
16	0.29	0.89
18	-	0.90
20	0.46	-
21	-	0.90
23	-	0.90
25	0.77	-
26	-	0.90
28	-	0.90
32	-	0.90
33	0.87	-
37	0.89	-
40	-	0.90
44	0.89	0.90
48	0.89	0.90
52	0.89	-
56	0.89	-

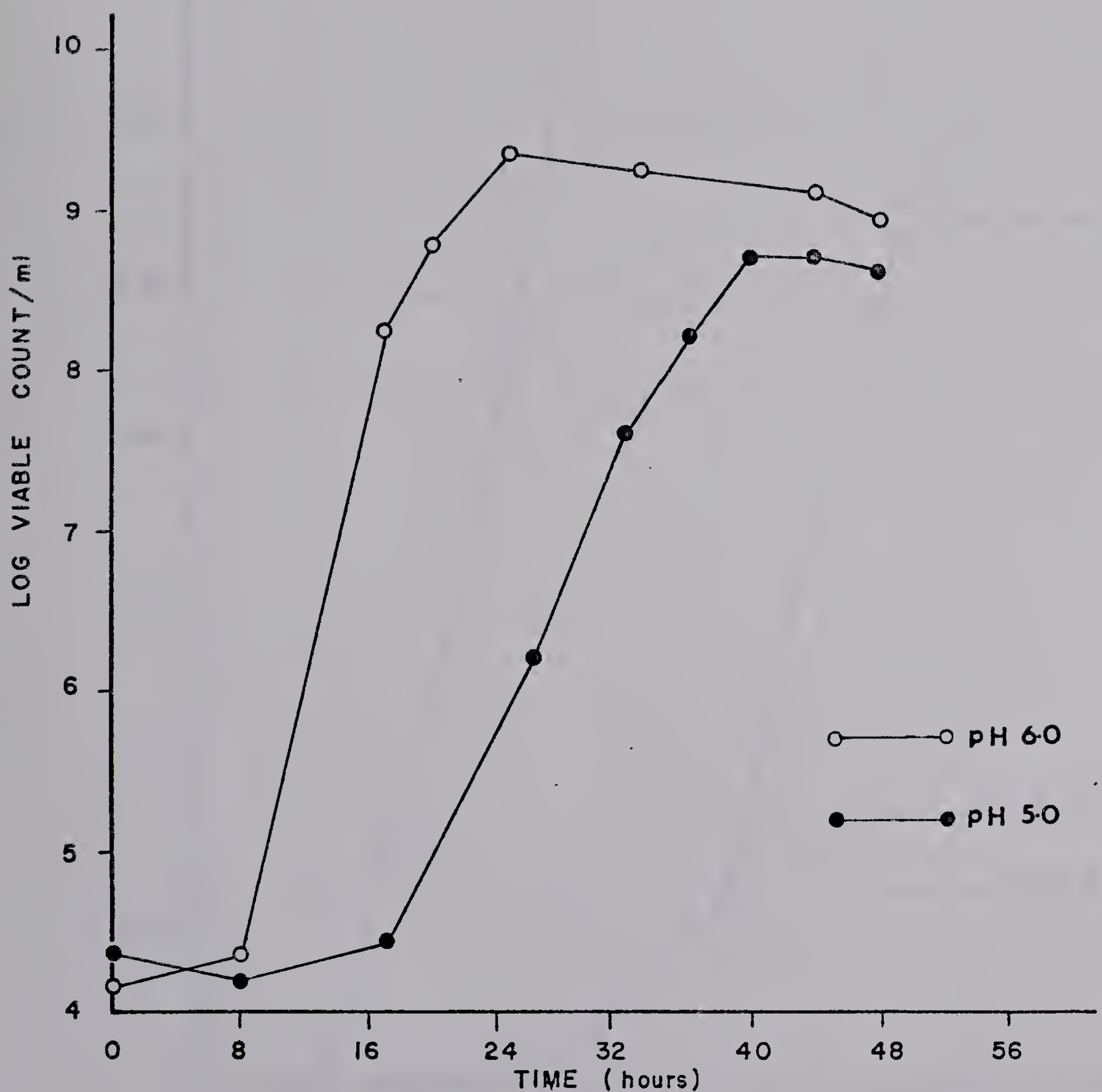

Fig. 14 Change in numbers of S. aureus in TSB of constant pH (5.0 & 6.0) at 25C

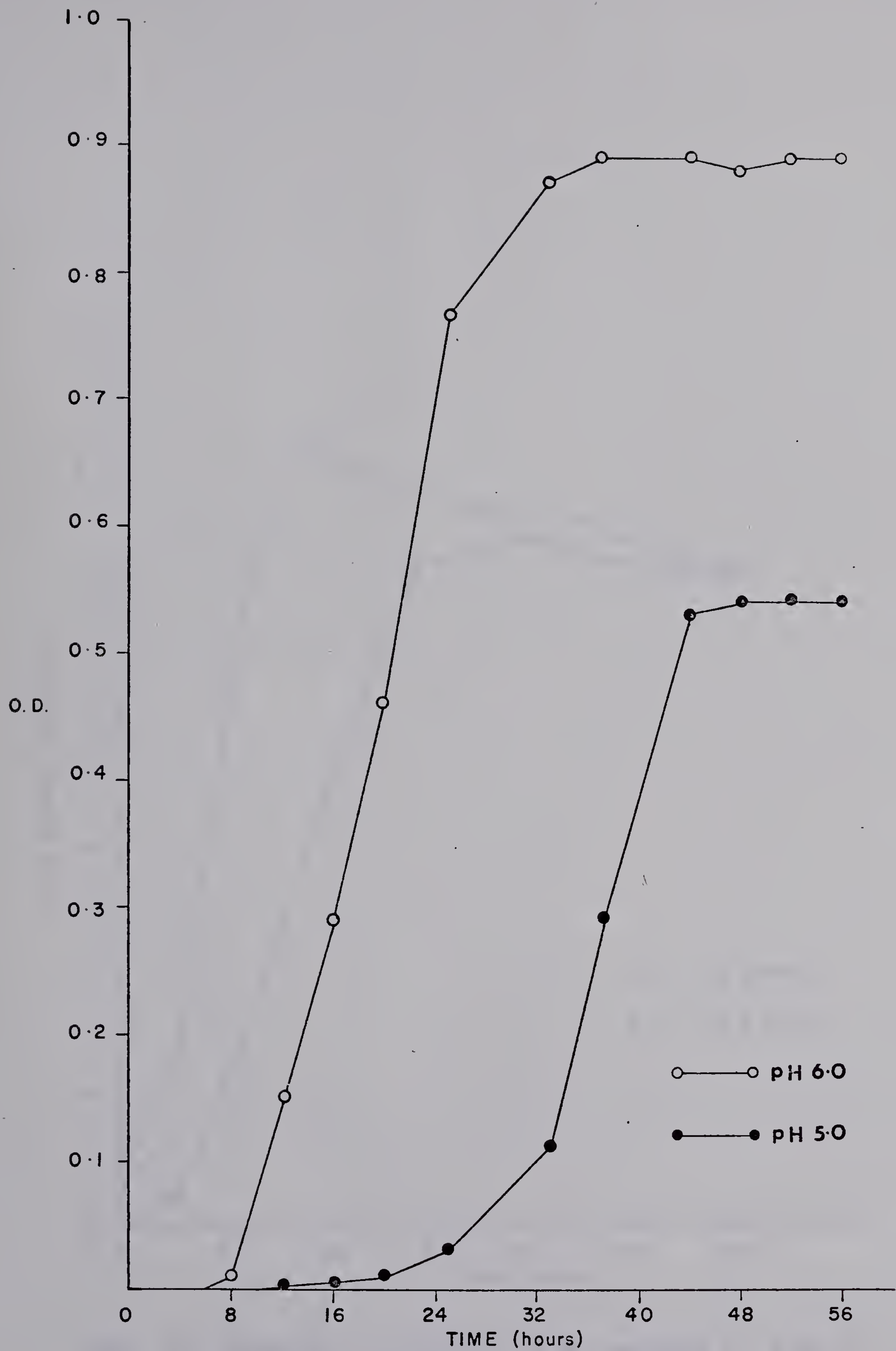

Fig. 15 Growth of S. aureus in TSB of constant pH (5.0 & 6.0) at 25C

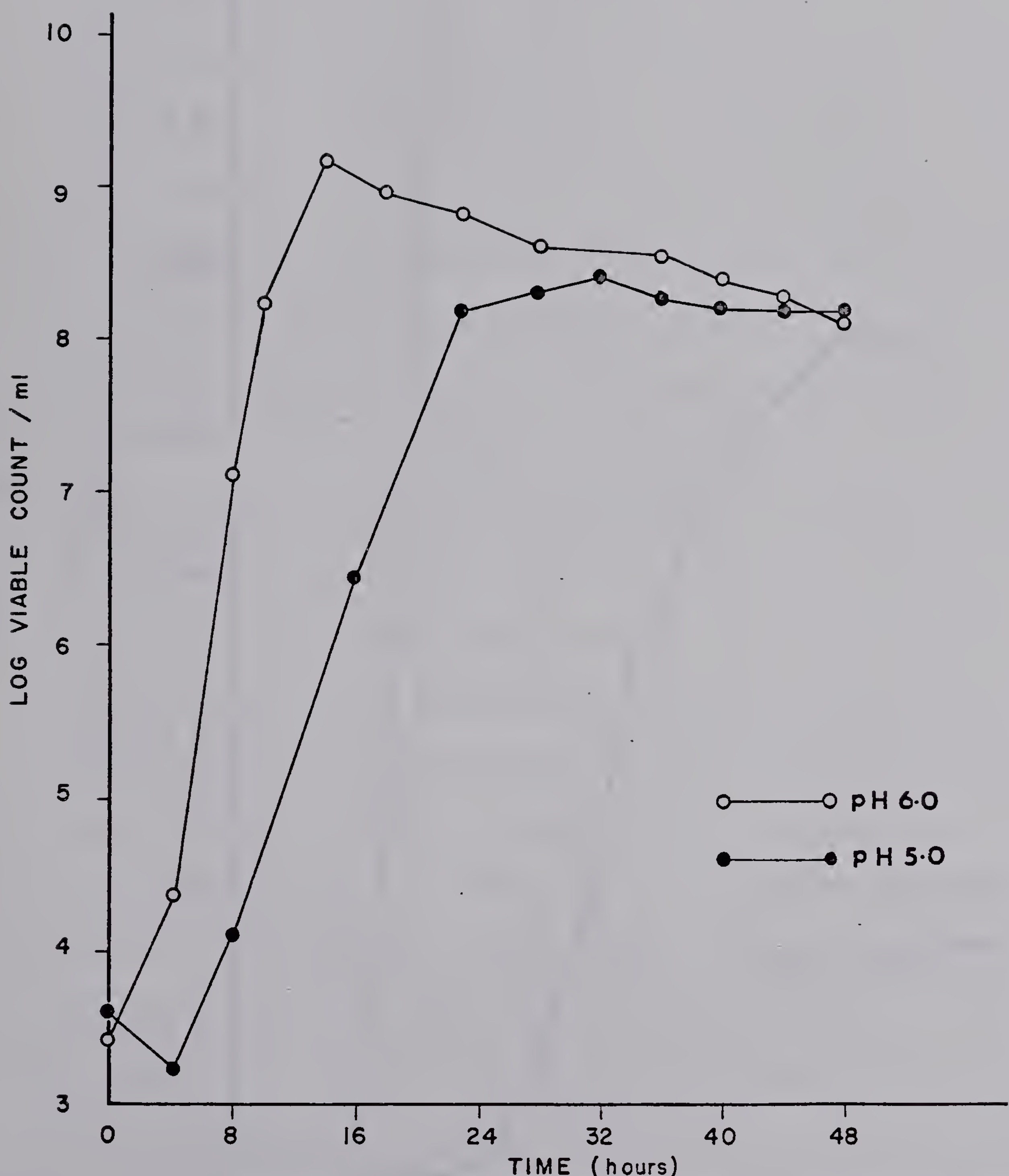

Fig. 16 Change in numbers of S. aureus in TSB of constant pH (5.0 & 6.0) at 37C

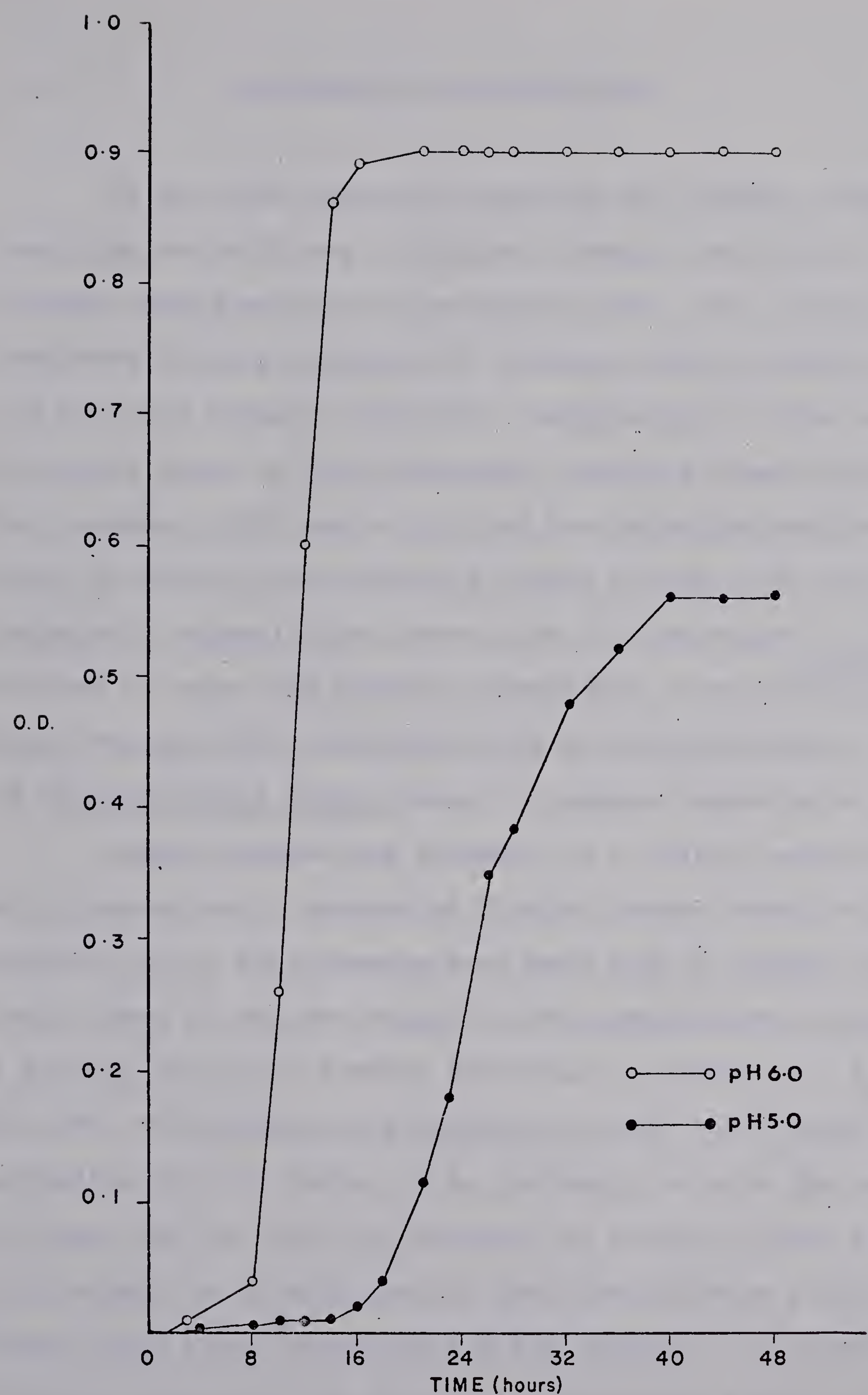

Fig. 17 Growth of S. aureus in TSB of constant pH (5.0 & 6.0) at 37C

DISCUSSION AND CONCLUSION

It has been generally accepted that Cheddar cheese made from raw milk has a distinct flavour from that of Cheddar cheese made with pasteurized milk. This flavour is preferred by many consumers of Cheddar cheese. However, as raw milk may contain pathogenic staphylococci, which are a potential cause of food poisoning, numerous cheese factories have changed their operations and are using pasteurized milk. In view of the potential danger of raw milk containing pathogenic staphylococci being used to make cheese it was decided to carry out several experiments in which cheese would be made from raw milk inoculated with an enterotoxigenic strain of <u>Staphylococcus aureus</u> known to produce enterotoxin B.

Cheddar cheese was prepared in a similar manner to the preparation of commercial Cheddar cheese except on a smaller scale. Five cheeses were made with <u>S. aureus</u> Cas 243 being added at various stages in the manufacturing process. A control was made in which there was no addition of <u>S. aureus</u> Cas 243. The cheeses were allowed to ripen for a period extending over 35 weeks. It is customary to cure the cheese at about 58F for ten days followed by curing at 45F. However, if the quality of milk used is poor then storage at 45F should take place immediately after hooping. The experimental cheeses were made from high quality milk and stored at 50F.

Conventional methods were used to prepare the cheese except in the preparation of the starter. It was found that

inoculation of skim milk and subsequent growth of the culture took place better at 32C than at 22C, the normal incubation temperature. Preliminary work with the starter showed that good acid development and coagulation of the milk took place in 18 hours when the starter was incubated at 32C. However, at 22C there was slow acid development,at least 44 hours being required to attain the amount of acidity desired. In some cases it took almost a week for coagulation to take place when incubated at 22C. This elevated temperature of incubation of the starter did not affect the making of the cheese as the product made was normal in flavour, texture, appearance and acid development.

From the data collected during the manufacture of the cheese it was evident that staphylococci compete with the starter organisms under normal conditions. There was an appreciable increase in numbers of staphylococci and although some of the organisms were lost in the expelled whey there was still a large number remaining in the curd. This is in agreement with the work carried out by Takahashi and Johns (1959).

The rate of multiplication of the organisms varied depending on the point of inoculation in the cheese making process. The highest rate (350x) was shown in cheese F in which the organisms were inoculated 12 hours before the cheese making operation. Although there was a very high multiplication rate during the making of this cheese, during the 12-hour holding period in which the milk was cooled by

running water (58F) there was no increase in numbers of staphylococci. The increased rate of multiplication in cheese F as compared to cheeses A, D and E (1.3, 22 and 8.2 times the original inoculum respectively) may be accounted for by the "adaptation" of the organisms during the 12-hour holding period. The increase in numbers of organisms continued in two cheeses, A and E, after hooping but otherwise there was a decline from this point on. Takahashi and Johns (1959) have indicated that the rate of multiplication in milk with a low SPC is much more rapid than in milk with a high SPC. The milk used for cheese F (total bacterial count 2.15×10^3 per ml) was low as compared to the milk used for cheese A (28×10^3 per ml) and it would appear as if there is some correlation of increase rate of staphylococci and original total count in the milk.

Plate count determinations showed that staphylococci die off during the ripening process but at no time did they disappear entirely. Some workers (Roughley and McLeod 1961 and Mattick _et al_. 1959) did not recover any coagulase-positive _S. aureus_ after curing the cheese for 22 weeks. However, others (McLeod _et al_.1962, Tuckey _et al_ . 1964 and Reiter _et al_.1964) have recovered coagulase-positive staphylococci after as long as 72 weeks of storage. In the present study coagulase-positive staphylococci were recovered after 48 weeks of storage and were still present in some of the cheeses in numbers (10^5) to suggest that they would

continue to be present for a longer time. Experiments to determine the coagulase activity of S. aureus Cas 243 after being in the cheese for over a period of 48 weeks showed that they were very active and retained their ability to coagulate plasma in a short time. The organisms are thus still a potential source of food poisoning and should the prepared cheese be used for making certain foods, which do not involve heating to a lethal temperature for the organism the danger of a food poisoning outbreak still exists.

Recent work has shown that staphylococci are commonly inhibited in competition with other microorganisms and in an environment such as that of Cheddar cheese the indigenous organisms would be more able to outstrip intruding organisms. In an effort to ascertain the effect of pH and three different temperatures (12, 25 and 37C) on the growth and production of toxin by S. aureus Cas 243 in TSB it was found that at a pH of 4.0 and a temperature of 12C growth was restricted, the numbers of organisms remaining fairly constant. At this same pH and higher temperatures (25 and 37C) the organisms gradually started to die off showing that these combinations of temperature and pH are unfavourable for growth. The effect of pH 5.0 and a temperature of 12C was the same as that of pH 4.0 at 12C. At 25 and 37C the organisms grew well in pH 5.0 but after a certain point when the pH dropped they started to die off. This effect was noticed after 16 hours of incubation. At a pH of 6.0 and a temperature of 12C staphylococci grew slowly at first but after a while began to grow profusely. It would appear

that this combination of temperature and pH had little effect on growth of the organism. At the higher temperatures of 25 and 37C staphylococci grew well until (like pH 5.0) the pH dropped to a point when the organisms started to die off (24 hours). At a constant pH of 5.0 and temperatures of 25 and 37C it was found that staphylococci increased in numbers beyond the maximum growth obtained with broth of the same pH but which was left unadjusted. Growth in broth of a constant pH of 6.0 and at temperatures of 25 and 37C was similar to that of pH 5.0 (constant). Examination of all broths showed that toxin was detectable in broths of pH 6.0 (constant and unadjusted) and at temperatures of incubation of 25 and 37C. Peters (1965) showed that staphylococci grew well in a pH range of 5.5 to 8.0 and that there was complete inhibition below pH 4.1 to 4.6. Staphylococci also grew well above pH 8.5 to 9.5 depending on the substrate and strain. Working with S. aureus Cas 243 he found that enterotoxin production was inhibited by an acid pH. Cultures with a pH below 5.0 produced little toxin.

Donnelly et al.(1966) have shown that it takes about 9 to 12 hours at 35C to produce staphylococcal enterotoxin in low count raw and pasteurized milk. A projection of this would infer that in the manufacturing process of Cheddar cheese the temperatures used and the period of time needed to make the cheese would not be enough to produce enterotoxin in large quantities. This of course would be dependent on the initial inoculum of the cheese milk. Serological determinations carried out on the cheeses made indicated that there was no detectable toxin. All of the

prepared cheeses were eaten by volunteers. No ill effects were observed.

From the results on growth and toxin production in cheese it would appear that if the pH of the cheese is normal, no toxin will be produced and the staphylococci will die out. The significance of the survivors, however, cannot be underestimated. The use of the cheese in the preparation of other food products in which the environmental conditions may change, such that the staphylococci can grow and produce toxin, is not advised.

The experiments using broth confirm the findings with the experimental cheese. Namely that at pH levels below 6 growth and toxin production are limited. It would be expected that toxin would be produced in broth at a lower pH than in cheese as a result of the more favourable environment in terms of competing organisms, aerobiosis, salt concentration and enzymic reactions.

It is concluded that cheese made under normal conditions from milk containing enterotoxigenic staphylococci will be safe for consumption provided that there is no pre-formed enterotoxin. Any factor that tends to raise the pH of the cheese, such as a slow starter or bacteriophage, may create conditions more suited to growth and toxin production by staphylococci. Any factor that tends to lower the pH of the cheese below normal will limit the growth of staphylococci but will not accelerate the rate at which they die off.

Finally, it is concluded that the presence of

enterotoxigenic staphylococci in cheese, even in large numbers, is not sufficient evidence to condemn the product as hazardous with regard to food poisoning. To condemn a product as hazardous in terms of food poisoning the presence of enterotoxin must be unequivocably demonstrated. However, a product containing a large number of staphylococci could be justifiably condemned with reference to the general practices and standards of hygiene expected in the food industry.

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